Avian Immunology

EDITED BY
Fred Davison, Bernd Kaspers
and Karel A. Schat

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To
Molly, Annegret and Laura

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FOREWORD

Chickens are not mice with feathers

Jim Kaufman

Immunology is not just restricted to investigations on the immune systems of mice and humans. Historically, studies on other species have contributed greatly to the development of immunological understanding. Amongst these other species, birds have provided an invaluable model for investigating basic immunological mechanisms. Birds have also played a crucial role in the development of vaccinology, and they still do to this day. The first attenuated vaccine discovered by Pasteur was directed against fowl cholera, a poultry disease. Likewise the first widely used vaccine against a naturally occurring cancer, Marek's disease, was developed for the poultry industry. The current threat from avian influenza reminds us of the necessity for gaining a thorough understanding of the avian immune system in order to develop novel and effective strategies for control.

Birds have many immunological mechanisms in common with mammals but have evolved a number of quite distinct strategies; they achieve the same goal through use of different mechanisms. Some of their different physiological characteristics such as their lung ventilation system, significantly different from the mammalian one, may partly explain the pressures for the evolution of different mechanisms.

One key feature of research on the avian immune system has been the seminal contributions it made on the key role of lymphocytes in adaptive immunity, graft-versus-host responses, delineation of the two major lymphocyte lineages – B cells and T cells – and gene conversion for developing the immunoglobulin repertoire. In addition the chicken major histocompatibility complex (MHC) – the first non-mammalian MHC to be sequenced – is compact, considerably smaller than its mammalian counterpart, and strongly associated with resistance to certain infectious diseases. No doubt the availability of the entire chicken genome sequence will help provide us with a more precise picture and a better understanding of the avian immune system.

Birds have been described as living dinosaurs, as exemplified by the discovery of dinobirds, mainly in China. This discovery provides a major incentive to carefully study the avian immune system and gain a better understanding of the rules of evolution. At the same time, we should bear in mind that infections provide an important selection pressure and immunocompetence is a valuable trait for the survival of a species. I firmly believe that a full understanding of the immune system will be acquired only through comparative analysis of its structure, function and physiology in a multitude of different species.

Apart from all these fundamental aspects of avian immunology, practical aspects should not be neglected. As consumption of meat is expected to increase considerably during this century (Delgado's livestock revolution) it must be expected that poultry will have a key contribution. Reproductive traits, a short productive lifespan, production of eggs, absence of dietary restrictions and worldwide distribution, all favour the use of poultry as a major source of animal protein. These same characteristics are also valuable for the avian research model. Well-defined inbred strains of chickens are already available. These can produce large numbers of progeny that are relatively easy to house and handle in laboratory research. From this point of view, chickens can be considered 'mice with feathers'.

This book on avian immunology is timely; it should play a key role by gathering together available information on the avian immune system and synthesizing new ideas to prepare for the future. I warmly congratulate the initiators on their achievement. They have persuaded some of the foremost world experts in avian immunology to review their respective subject areas, condense their thoughts and share with the reader new ideas about the immune system. There has never been a more exciting time to read about and study avian immunology.

Paul-Pierre Pastoret
Fontin, Belgium
January 2007

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1 THE IMPORTANCE OF THE AVIAN IMMUNE SYSTEM AND ITS UNIQUE FEATURES

FRED DAVISON

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INTRODUCTION
THE CONTRIBUTION FROM AVIAN LYMPHOCYTES
THE CONTRIBUTION OF THE BURSA OF FABRICIUS
THE CONTRIBUTION OF THE CHICKEN MHC
THE CONTRIBUTIONS TO VACCINOLOGY
CONCLUSIONS
REFERENCES

INTRODUCTION

The avian immune system provides an invaluable model for studies on basic immunology. Birds and mammals evolved from a common reptilian ancestor more than 200 million years ago and have inherited many common immunological systems. They have also developed a number of very different and, in some cases, remarkable strategies. Due to their economic importance, and the ready availability of inbred lines, most avian research immunology has involved the domestic chicken, *Gallus gallus domesticus*. A remarkable consequence of this research has been the seminal contributions it has made to understanding fundamental immunological concepts, especially the complete separation of developing bursa (B)- and thymus (T)-dependent lymphocyte lineages. Some of the observations were made by chance, while others resulted from painstaking work which took advantage of special avian features, for instance the ease of access to, and precise timing of, all the stages in embryonic development. Some of the avian findings were described before being recognized as important and subsequently explained in mainstream immunology. The story of avian immunology is fascinating and by no means complete, as there is still the need for explanations of a number of unique features and the different strategies adopted by the avian system. In this chapter some of the "firsts", rightly attributed to avian immunology, are described and the importance of further studies in avian immunology highlighted.

THE CONTRIBUTION FROM AVIAN LYMPHOCYTES

The advent of modern cellular immunology, and the fundamental role that lymphocytes play, is generally credited to the 1950s and 1960s, when lymphocyte function became an active subject for research (Burnet, 1971). The immunological significance of lymphocytes emanated from

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some seminal studies carried out by Gowans, Chase and Mitchison, Simonsen and their contemporaries (see Gowans, 1959; Burnet, 1971). In elegant experiments using laboratory mammals, these workers demonstrated using cell transfers that lymphocytes are essential for generating immune responses and retaining memory of previous exposure to an antigen. However, evidence that lymphocytes play such a key role in protection against infection and in tumour rejection had been in existence for almost 40 years (Murphy, 1914a, b, 1916, 1926), though little attention had been paid to it (Silverstein, 2001). This was almost certainly because, at the time that they made, the observations could not be properly explained and, possibly, because the experimental animal involved was the chicken.

Between 1912 and 1921, James Murphy, an experimental pathologist working at the Rockefeller Institute for Medical Research in New York, performed a series of remarkable experiments using chickens and their embryos to study the growth and rejection of tumour grafts. His experiments appeared to prove beyond question that the lymphocyte is the active component in tissue graft rejection, in protection against infection and, by implication, in innate and acquired immune responses (Silverstein, 2001). Murphy (1914a) observed that the fragments of rat tumours would not grow in the adult chicken, just as they did not grow in other species (xenogenic rejection). However, they could be grown on the chorioallantoic membrane (CAM) of developing chick embryos, although only up to about 18 days incubation. In older embryos tumour grafts were rejected, just as they were if grafted onto the newly hatched chick or an adult bird. Interestingly, Murphy (1914a) observed that the grafts which grew on embryos could be transferred to fresh embryos without any evidence of them being altered. They also retained their tumorigenic capacity if re-grafted onto a rat. Murphy (1914a) commented that these cellular changes occurring when living tissue is grafted onto an unsuitable host are the same, regardless of the type of host resistance, be it natural resistance because of species differences (allogeneic or xenogeneic rejection) or acquired immunity due to recovery from a tumour implanted earlier. The histological picture consisted of oedema surrounded by fibroplasia in the host tissue, the budding out of blood vessels and infiltration of surrounding host tissues with small lymphocytic cells. The inevitable consequence was that cells in the graft died fairly quickly leaving only a scar. These were quite profound though, at the time, unappreciated observations.

Murphy (1914b) also performed a series of elegant experiments using adult chicken tissues co-grafted onto the CAM with fragments of rat tumours. He observed that chicken tissues containing an abundant supply of lymphocytes, such as the spleen or bone marrow, caused tumour grafts to be rejected; whereas these were not rejected if the co-grafted tissue lacked a rich supply of lymphocytes. Later on, Murphy (1916) showed that after grafting fragments of adult chicken spleen onto the CAM of a 7-day embryo, the embryo's own spleen became grossly enlarged (splenomegaly). This is the first published record of a graft-versus-host response (GvHR). Much later, it was explained by Simonsen (1957) as the immunologically competent lymphocytes of the adult responding to mismatched major histocompatibility complex (MHC) molecules expressed by the embryonic cells. The embryonic cells were recognized as foreign causing the adult cells to replicate and destroy the embryonic host's lymphocytes. Graft-versus-host disease, in which allogeneic bone marrow transplants recognize the tissues of the graft recipient and cause severe inflammatory disease, is a major problem for immunosuppressed recipients receiving bone marrow transplants. The phenomenon became a major concern with the introduction of human bone marrow transplantation. Nonetheless, the phenomenon was first described using chick embryos (Murphy, 1916).

THE CONTRIBUTION OF THE BURSA OF FABRICIUS

Without doubt, the most significant contribution that avian immunology has made to development of mainstream immunology was in delineating the two major arms of the adaptive immune system. As already pointed out, in the 1960s the significance of lymphocytes was just becoming appreciated and it was generally accepted that there are two types of adaptive

immune responses: humoral responses involving antibodies and cellular responses mediated by macrophages and lymphocytes. Since antibodies are produced by plasma cells, which themselves are derived from lymphocytes, it was not understood how such lymphocytes could be of the same type as those cells involved in cell-mediated functions. The bursa of Fabricius, an obscure sac-like structure attached to the proctodeal region of the bird's cloaca (Plate 1.1), played a crucial role in unravelling this problem.

The cloacal bursa, takes its name from Hieronymus Fabricius of Aquapendente (1537–1619), also known as Girolamo Fabrizi d' Acquapendente (Fig. 1.1). He was Professor of Surgery at the University of Padua, Italy from 1565 to 1613 (Adelmann, 1942) and by all accounts a brilliant anatomist, embryologist and teacher. For his pioneering work he was later credited in Italian medical science as the "Father of Embryology". Fabricius not only carried out dissections on human cadavers but also extended his anatomical studies to other species, providing most beautiful detailed drawings of his work. From his observations of avian anatomy he surmised that the cloacal bursa, a hollow structure connected by a duct to the proctodeal region of the cloaca in the hen, most likely acts as a receptacle for storing semen:

> Since the sac is pervious, so that there is an open passage from the anus to the uterus itself and another from the uterus to the sac, that is, above and below, and since it is closed at the other end, I think it is the place into which the cock introduces and delivers semen so that it may be stored there (from Adelmann, 1942).

This is not the case, however, but the role of the cloacal bursa continued to puzzle researchers over the following 350 years. Some surmised that, since the bursa of Fabricius regresses with sexual maturity, its size having an inverse relationship with the size testes and the adrenals, it must be some sort of endocrine or lymphoid gland associated with growth and sexual development (Glick, 1987).

FIGURE 1.1 Hieronymous Fabricius of Aquapendente, Professor of Surgery at the University of Padua from 1565 to 1613. Fabricius was the first to describe the bursa, which is now known to be a primary lymphoid organ, attached by a duct to the proctodeum in birds and essential for the development of bursal-derived (B) lymphocytes and the avian antibody repertoire.

Over the years many investigated the function(s) of the bursa including one young researcher, Bruce Glick, working at the Poultry Science Department at Ohio State University, USA. Glick surgically removed bursas from young chicks to investigate the effect on growth. By chance, after one experiment was concluded a colleague, Timothy Chang, asked if he could use some of the birds for a class demonstration on antibody production. A group of the chickens was injected with *Salmonella* spp. "O" antigen but 1 week later, when the class carried out tests with blood and antigen, there was no evidence of agglutination. Chang, somewhat perplexed, reported the failure to Glick who was able to identify the non-responder chickens as those that had been bursectomized. At the time they did not seem to fully appreciate the singular importance of this finding (Glick, 1987), but were able to confirm their initial observations in further experiments and wrote up a paper entitled: "The role of the bursa of Fabricius in antibody production." This was submitted to *Science* but rejected on the grounds that further elucidation of the mechanisms was necessary before the paper could be accepted for publication (see Glick, 1987). The paper was subsequently submitted to *Poultry Science* (Glick *et al*., 1956), where, for a time, it failed to draw much attention. Several years passed before the significance of the work was properly appreciated and mainstream immunologists took an interest in the chicken's immune system. It was eventually concluded that the avian bursa must be essential for antibody-mediated immunity, whereas the thymus, which also undergoes involution during sexual development, is necessary for cell-mediated immunity (Szenberg and Warner, 1962; Warner and Szenberg, 1962; Warner *et al*., 1962). Almost a decade after Glick and Chang's initial observations (Glick *et al*., 1956), Cooper *et al*. (1965) published their seminal paper on the delineation of the bursal and thymic lymphoid systems in the chicken. These workers proposed that, because of the similarities in the lymphoid tissues and immune systems of birds and mammals, a mammalian equivalent for the bursa of Fabricius must exist and provide a source of B-dependent lymphocytes to make antibodies. Later this bursa equivalent was identified as bone marrow. The division of the adaptive immune system into B- and T-dependent compartments has remained a central tenet of immunological thinking ever since. The term B lymphocyte is derived from "bursa-derived lymphocyte" in honour of that peculiar avian lymphoid structure which provided the original evidence.

Gene Conversion and the Bursa

Antibodies recognize specific conformational molecular shapes on their target through the immunoglobulin (Ig) variable region. Antigenic shapes are legion so an immunocompetent individual must be capable of generating an antibody repertoire with a huge number of Ig specificities (Janeway *et al*., 2001). Different B cells produce Ig molecules of different specificities and each B cell is capable of producing only one Ig specificity. In man and mouse the antibody repertoire of B cells is generated by a process known as Ig gene rearrangement, which is ongoing throughout life. In the case of the Ig light chain, genes that encode the variable region (V_L gene segment), a joining region (J_L gene segment) and the constant region (C_L gene segment) are spatially separated by non-coding segments in germline DNA (Plate 3.4). Non-coding segments are spliced to allow the joining of V_LJ_L regions in genomic DNA, while excision of the non-coding section in the RNA transcript allows the $V_LJ_LC_L$ regions to combine permitting translation of a functional Ig light chain molecule (Tonegawa, 1983). In the case of the Ig heavy chain a further diversity (D) gene is involved, making a V_HDJ_H rearrangement, otherwise the process is similar. Since multiple copies of V, J and D genes exist in the mammalian Ig locus: (1) random recombination of the different gene segments; (2) different combinations of heavy and light chains paired together; diversity introduced at the joints between the different gene segments by the variable addition and subtraction of nucleotides; and finally (3) point mutations introduced into the rearranged sequence diversifying these region further (somatic hypermutation), allow for a vast ($\sim 10^{11}$) antibody repertoire to be generated (Janeway *et al*., 2001).

By contrast the cluster of genes encoding the chicken Ig light chain has only a single copy of the functional V_L and J_L genes (Reynard *et al*., 1985). Hence diversity due to V_LJ_L joining is very limited and the effects of VJ rearrangement are minimal (a detailed description can be

found in Chapter 4). Likewise with the Ig heavy chain locus, the presence of single functional V_H and J_H genes means that little diversity can be generated through V_HDJ_H rearrangement. However, clusters pseudogenes, upstream of the heavy and light chain Ig loci have a critical role in the generation of chicken antibody diversity. By a process known as somatic gene conversion, V_L and V_H sequences are replaced with pseudogene sequences. An enormous amount of diversity is generated by substantial diversity in the hypervariable regions of the donor V pseudogenes and somatic gene conversion events accumulate within single functional V_L or V_H genes. Perhaps, chickens represent the extreme situation with only one functional V_L gene while other species such as the duck have up to four functional V_L genes, although they still use gene conversion to introduce variability. It seems that, uniquely, birds rely on somatic gene conversion for generating an antibody repertoire which is the equal of that in immunocompetent mammals. Interestingly, it has recently been shown that if gene conversion is blocked in chicken B cells then somatic hypermutation occurs instead (Arakawa *et al*., 2002). It has also been observed that gene conversion is not just limited to birds. Gene conversion also occurs in rabbits (Becker and Knight, 1990), pigs and other mammalian species, though none appear to rely on it as the exclusive means of generating the antibody repertoire.

The striking fact about avian somatic gene conversion is that it only occurs in the bursa of Fabricius (see Chapter 4). For instance, if the bursa is destroyed early in development (60 h), then those chicks that hatch produce only non-specific IgM and are unable to mount specific antibody responses. In other words, they do not have an antibody repertoire and are incapable of eliciting typical responses or isotype switching to produce IgG or IgA. Whereas, if the bursa is removed much later during embryonic development, but before 18 days when the B lymphocytes have begun to migrate from the bursa into the peripheral lymphoid tissues, then the hatched chicks lack circulating Ig and are incapable of eliciting specific antibody responses. We know that pre-bursal stem cells enter the bursa between 8 and 14 days incubation and have already undergone gene Ig rearrangement, probably in the embryonic spleen and bone marrow, for they express IgM on the surface (see Chapter 4). Within the bursa they undergo rapid rounds of cell division and only within this unique environment does gene conversion occur. In the absence of the bursa an antibody repertoire cannot be generated and a major arm of the immune system becomes non-functional. The chicken antibody repertoire is generated during the late embryonic stage and for a short period after hatching. As the chick ages so its B cells undergo additional rounds of somatic gene conversion and the antibody repertoire becomes expanded until a mature repertoire is achieved around 5–7 weeks when the bursa is fully mature. Thereafter, the bursa begins to regress as sexual maturity approaches and the adult probably relies on post-bursal stem cells in the bone marrow as the source of B cells.

Of course, generating the antibody repertoire in a burst of activity in the young animal has its risks. Any pathogen which targets and destroys bursal cells will have a devastating effect on antibody-dependent immune responses. One such virus is the small RNA virus that causes infectious bursal disease. Infection of the neonate chick with infectious bursal disease virus (IBDV) may cause no clinical disease but destroys bursal B cells leaving the chick incapable of mounting an antibody response to other pathogens, although paradoxically there is a good response to IBDV itself (see Chapter 16). The insidious nature of IBDV leaves the chick vulnerable to opportunistic infections, and unprotected by subsequent vaccinations. So relying on the generation of the antibody repertoire in a single location and over a relatively short-time span is not without its hazards and, perhaps, represents one of the more "risky" strategies birds have adopted.

THE CONTRIBUTION OF THE CHICKEN MHC

Pathogens are diverse, cunning and occupy different niches within the body of the host. Apart from pathogens that are found outside of cells, such as *Clostridium*, *Escherichia coli* and *Bordatella*, there are intracellular pathogens that can be found in the cytoplasm or in cellular vesicles. In addition, retroviruses and herpesviruses integrate into the host's own genome.

To match this diversity, and the different locations where pathogens are found, higher vertebrates have evolved a number of different innate and adaptive immunological mechanisms to improve the chances of survival. Antibodies recognize conformational epitopes on the pathogen's molecules but need to come into direct physical contact in order to neutralize a pathogen, as well as recruit cells and other molecules that bring about disposal. Ig molecules are large and cannot easily enter a viable cell, so an intracellular pathogen cannot be recognized by an Ig molecule unless the pathogen's molecules are expressed on the surface of that infected cells. However, the cellular immune system has evolved a number of methods for recognizing and destroying cells with intracellular pathogens. These mechanisms allow the host's effector lymphocytes to recognize infected, or neoplastic, cells through the expression of proteins that have been proteolysed within the cell and are expressed on the cell surface as peptide fragments. In mammals the molecules that present peptides on the surface of cells are encoded by a highly polymorphic genetic region known as the MHC.

The MHC region was originally recognized through its effects on tissue graft rejection and, of course, GvHR. Two major types, or classes, of MHC molecules are encoded by genes in the mammalian MHC region and expressed on the surface of cells, class I and II MHC molecules (more fully described in Chapter 8). Although both types of molecules are heterodimers: the class I MHC molecule consists of an α-chain encoded by the MHC together with an invariant β_2-microglobulin molecule from a gene outwith the MHC locus; the class II molecule consists of two peptide chains (α and β) whose genes are found in the MHC region. Unlike the MHC class I heterodimer that is present on most types of cells, expression of class II MHC molecules is restricted to antigen-presenting cells such as macrophages, dendritic and B cells. During synthesis inside the cell both classes of MHC molecules trap peptide fragments in a cleft on, what is to become, the outer surface of the extracellular domain of the MHC heterodimer. On reaching the cell surface these peptides are displayed as peptide:MHC complexes to signal T cells through their T cell receptors. Since even the smallest virus produces a number of proteins, and large pathogens can produce hundreds, there needs to be a large repertoire of T cell receptors expressed by different T cells in order to recognize the multiplicity of peptide fragments derived, not from the host's own cells, but made by pathogens or neoplastic cells. This T cell repertoire is developed within the thymus (described in Chapter 5), where developing T cells with receptors that recognize self-peptides associated with MHC molecules are eliminated, or inactivated, before they mature so as to prevent self-recognition and autoimmunity. Mature T cells capable of recognizing "foreign" peptides are released from the thymus into the periphery and become activated if they recognize peptide fragments expressed on MHC molecules. Interestingly, the T cell repertoire in the chicken is developed in the thymus in a similar way to that of mammals. There is no evidence for a somatic gene conversion mechanism such as that occurs in the avian bursa.

In mammals the MHC is a large and complex region that contains much redundancy (Trowsdale, 1995). In the human, it consists of about 4 million base pairs encoding at least 280 genes. Separate regions contain several MHC class I and II genes (there are a vast number of alleles) that are highly expressed on cells. These regions are separated by third region that encodes immune response genes (class III). Humans express two or three class I molecules and three or four class II molecules that are highly polymorphic. The high polymorphism is probably driven by the ever-changing variations in pathogens (Doherty and Zinkernagel, 1975; Zinkernagel and Doherty, 1979), although different haplotypes appear to confer approximately the same degree of protection against most of the infectious pathogens. In Chapter 8, Kaufman points out that the associations between the human MHC and infectious disease are, actually, very slight.

The chicken MHC is known as the B locus, since it was first identified as a serological blood group locus (Briles *et al*., 1950) encoding the polymorphic, and highly immunogenic BG antigen. This BG antigen is highly expressed on blood cells and has no known mammalian equivalent. Later it was shown that the B locus must constitute the avian MHC, because of its strong association with cell-mediated immune functions, such as graft rejection, mixed lymphocyte reactions and GvHR. Remarkably, and in marked contrast to the large mammal MHC, the chicken B locus is minute, spanning only 92 kilobases and encoding 19 genes and making it

approximately 20-fold smaller than the human MHC (Kaufman *et al*., 1999). Only two copies each of class I (BF) and class IIβ (B/Lβ) genes are found within the chicken B locus. The marked differences between the chicken MHC and its mammalian counterpart have led Kaufman to argue (see Chapter 8) that the chicken B locus represents a minimal essential MHC that must have evolved after birds and mammals separated some 200 million years ago. Another striking feature of the chicken minimal MHC region is that, not only does it affect a number of important cell-mediated immune functions, but also determines life or death in response to a number of pathogens (Kaufman *et al*., 1995; Kaufman, 2000). In Chapter 8, Kaufman develops the argument that chickens, with a minimal essential MHC, appear to have adopted a completely different strategy to that of mammals whose MHC is large and complex. The close association between the chicken MHC and disease resistance is fascinating and at first sight seems to be a suicidal strategy. However, many lessons can be learned from studying chicken immunology and its parallel evolution. By studying the minimal chicken MHC, light should be thrown on the important interactions between pathogens and the immune system and the importance of different evolutionary strategies.

THE CONTRIBUTIONS TO VACCINOLOGY

In any review of the "firsts" credited to avian immunology tribute needs to be paid to pioneering developments in the practical uses of immunology; in other words, the use of vaccination. The modern poultry industry relies heavily on vaccines to protect against a wide range of different infectious agents. Vaccinations are frequent and begin from the day of hatching or even before. Chickens are immunized with live-attenuated vaccines and killed vaccines delivered by various routes (injection, aerosol spray, drinking water, etc.) in mass vaccination programmes that dwarf programmes in human medicine.

Every biology student knows that Edward Jenner is the founding father of vaccination. Jenner discovered that cowpox pustules, obtained from an infected milkmaid, protected an 8-year-old boy, James Phipps, against the related smallpox virus. However, further developments in vaccination, and indeed the term vaccination, only came into use about a century later, following studies by one of the greatest nineteenth century scientists, Louis Pasteur. Here again the chicken had a privileged role and serendipity played its part.

In 1878, Pasteur was investigating chicken cholera, a disease with devastating effects, causing chickens to become anorexic, moribund and usually leading to their death. Pasteur investigated the causative agent, now known as the *Pasteurella multocida*, and succeeded in growing the bacteria in culture. The story goes (De Kruif, 1953) that Pasteur's research was interrupted by a holiday and a culture was left in a flask in the laboratory. Upon resuming his research, Pasteur inoculated chickens with this stale culture. The chickens became sick but then recovered within a few days. We now know that the bacteria had become attenuated and no longer capable of causing mortality. Pasteur did not know this and decided to inject new chickens with a fresh bacterial culture but, unfortunately (or fortunately!), his assistant found that chickens at the local market were in short supply. A number of fresh birds were obtained but those that had recovered from the inoculation with the stale culture needed to be reused. As expected the new chickens all succumbed to the fresh pathogen and died but those that had recovered from the previous treatment with stale inoculum again recovered. Pasteur realized he had achieved with chicken cholera what Jenner had accomplished with smallpox some 100 years earlier, only in this case he had attenuated (weakened) the pathogen by prolonged storage. He called the attenuated culture "vaccine" (Pasteur, 1880) in honour of Edward Jenner and then began a, largely successful, search for similar vaccines against other infectious diseases such as pig erysipelas, sheep anthrax and rabies. The serendipitous discovery of attenuation was another novel finding made using the chicken. Since then the development of vaccines has had far-reaching implications for the health and welfare of both humans and domesticated animals. The search for better, more effective vaccines still goes on apace.

Another first in vaccine development was in the control Marek's disease (MD) a naturally occurring neoplastic disease of chickens. MD became the scourge of the poultry industry in the 1950s and 1960s, causing major problems for animal health and welfare and becoming a huge financial burden. Before the introduction of MD vaccines, morbidity and mortality in laying flocks ranged from 0% to 60% or greater, with losses of 30% being common (Powell, 1986). The development of an MD vaccine represents the first example of widespread use of vaccination to protect against a natural form of cancer (Purchase, 1973). Over the years it has been remarkably effective (Witter, 2001), although not without problems.

MD was first described as a neurological disease (polyneuritis) by Josef Marek (1907). The condition caused paralysis of the wings and legs and was associated with mononuclear infiltrations and enlargement of the major nerves. Later it was observed (Pappenheimer *et al*., 1926, 1929) that in addition to lesions in the nerves and central nervous system, chickens also developed lymphoid tumours in several visceral tissues (visceral lymphomatosis) such as the ovary, liver, kidneys, adrenal and muscles. With intensification of poultry production the acute form of MD became a dominant feature. Although the introduction of MD vaccines in the 1970s controlled the disease, in some countries problems with vaccine breaks have continued to occur with regularity and there is now good evidence that the causative herpesvirus (Marek's disease virus, MDV) has been able to evade vaccine-induced immune responses by evolving to greater virulence. Since the 1980s, the response of the industry in some countries has been to introduce more aggressive vaccine strategies, using "hotter" vaccines, such as CVI988, either alone or in combination with other MD vaccines (bivalent or trivalent combinations). The most efficacious current MD vaccine CVI988 is derived from a serotype 1 MDV that is weakly oncogenic in genetically susceptible chickens and this has led some to raise the important question (Witter, 1997): Where do we go if hypervirulent MDV pathotypes evolve that can break through the protection of CVI988?

MDV is not the only example of a poultry virus that has changed in response to the introduction of widespread use of vaccines. More virulent isolates of another lymphotropic virus, IBDV were isolated in the late 1980s. IBDV is a small double-stranded RNA virus that encodes only five viral proteins. As already pointed out, IBDV targets B lymphocytes in the bursa of young chicks causing no clinical signs in neonates but causing clinical disease and some mortality in older chicks (see earlier). Chicks are protected by maternal antibodies derived via the yolk, but it became clear in the late 1980s that the very virulent IBDV being isolated from outbreaks was capable of causing disease in the presence of high levels of maternal antibodies. The response of the industry has been to introduce more aggressive ("hotter") vaccines to protect against the more virulent IBDV strains that have evolved under the pressure of vaccine use. The risk, however, is that these hotter vaccines could themselves be capable of causing bursal damage and immunosuppression in chicks that are poorly protected by maternal antibodies or have a susceptible genotype. Here again we have an example of a strategy that is holding at present but may not be sustainable long term. More aggressive vaccines or vaccination regimes cannot be introduced without the risk that the vaccines themselves could be harmful.

These issues have been addressed in epidemiological studies on implications of the use of vaccines on the evolution of pathogen virulence. Researchers (Gandon *et al*., 2001) were chiefly concerned with the use of different vaccines developed against the malaria parasite and implications for human populations. Mathematical modelling was carried out based on the premise that most vaccines are imperfect and rarely provide full protection from disease. Using various models, these authors predicted that vaccines designed to reduce the growth and/or toxicity of pathogens actually diminished selection pressure against more virulent pathogens. Consequently, subsequent pathogen evolution may lead to higher levels of intrinsic virulence and hence more severe disease in unvaccinated individuals. Such evolution would tend to erode any population-wide benefits, such that overall mortality could increase with the level of vaccination coverage. Interestingly, the authors found evidence of this phenomenon in the practical problems arising from MD vaccination. Current MD vaccines target viral replication and do not prevent infection with MDV, consistent with mathematical modelling that predicts evolution

of pathogen virulence. Yet again, evidence from work on the chicken has proved to be the pathfinder and pointed to important problems to take account of in vaccine design.

Embryonic (*In Ovo*) Vaccination

The problems associated with challenge from virulent MDV when chicks are moved into rearing quarters, the vast numbers of chicks requiring vaccinations at the hatchery, as well as the occasional failures caused by manual vaccination, has led to a search for new ways for mass vaccination that can be done at an even earlier stage than the day-old chick. Sharma and colleagues at the East Lansing Regional Poultry Laboratory, USA, demonstrated that chick embryos could be successfully vaccinated against MDV at 17–18 days incubation (Sharma and Burmester, 1982; Sharma and Witter, 1983). The automated INOVOJECT® system, which allows the automated mass application of vaccines to large numbers of eggs (up to 50 000 eggs per hour, Gildersleeve *et al*., 1993) was developed and has been widely applied in the poultry industries of some countries (for a fuller description, see Chapter 20). In the USA, almost all broilers (approximately 7 billion per year) are vaccinated by this method. *In ovo* vaccination is achieved by puncturing a small hole through the blunt end of the egg with an oblique pointed needle then passing down a smaller needle to deliver a small amount (usually 50 μl) of vaccine into the amniotic cavity. Since the amniotic fluid is imbibed prior to hatching the vaccine is then taken up by the embryo. Interestingly, the reasons why *in ovo* vaccination, applied to such an immunologically immature individual, is so effective remains to be fully explained. Nevertheless, the finding that a higher vertebrate can be protected against challenge early after hatching (birth) by vaccinating the embryo is quite revolutionary and its practical application in mass vaccination a great achievement. The immunological explanations will surely come in due course.

CONCLUSIONS

Compared to mouse and man the avian immune system may seem like a poor relation and yet it has provided important insights into fundamental immunological mechanisms and can claim a number of important "firsts" especially with regard to vaccinology. From the immunological viewpoint, the chicken is, perhaps, the best-studied non-mammalian species. The recent publication of the chicken genome (International Chicken Genome Sequencing Committee, 2004) has provided the opportunity for a "quantum shift" in the search for new chicken genes and exciting possibilities for developing new tools and reagents to study immune responses and immunogenetics. Interest in studying immune responses of other avian species is increasing, including species of wild birds. Ecologists are now taking an interest in measuring immunocompetence and determining its importance as a heritable trait for the survival, both of the individual and the population. Avian immunology is a fascinating and growing field and will surely provide new and exciting insights for mainstream immunology in the future.

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2 STRUCTURE OF THE AVIAN LYMPHOID SYSTEM

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INTRODUCTION
THE GC OF PERIPHERAL LYMPHOID ORGANS
THYMUS
BURSA OF FABRICIUS
SPLEEN
GUT-ASSOCIATED LYMPHOID TISSUES
HARDERIAN AND CONJUNCTIVAL-ASSOCIATED LYMPHOID TISSUES
MURAL LYMPH NODE
ECTOPIC LYMPHOID TISSUE AND THE PINEAL GLAND
BONE MARROW
BLOOD
REFERENCES

INTRODUCTION

Understanding the physiology and immunology of the lymphoid system is handicapped without knowledge of its basic structure. Lymphomyeloid tissues develop from epithelial (bursa of Fabricius and thymus) or mesenchymal (spleen, lymph nodes and bone marrow) anlages which are colonized by blood-borne haematopoietic cells. In the case of central lymphoid organs, haematopoietic stem cells enter the bursal or thymic anlages and develop to become immunologically competent B and T cells, respectively. Hence, B and T cells are of extrinsic origin, as proposed by Moore and Owen (1965, 1967) and described in more detail in Chapter 3.

Immunologically mature cells enter the circulation and colonize the peripheral lymphoid organs: spleen, lymph node and gut-, bronchus- and skin-associated lymphoid tissues. This peripheralization process can be experimentally manipulated by surgical or chemical interventions. The homing of the lymphocytes occurs through high endothelial venules (HEV), which are located in the T-dependent zones of peripheral lymphoid organs. In these organs B and T cells occupy different compartments, referred to as B- and T-dependent zones, respectively. In the avian spleen the T-dependent zone – called peri-arteriolar lymphatic sheath (PALS) – surrounds the splenic central artery, whereas in other lymphoid organs this is not as well defined, and the interfollicular region is regarded as the T-dependent area. B cell compartments are the germinal centres (GC). In addition to the GC the peri-ellipsoidal white pulp (PWP) of the spleen is also a B-dependent region, like the marginal zone of the mammalian spleen. Possibly, this

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B cell population in the PWP is responsible for production of antibodies against bacterial capsular antigens (pneumococcus antigen). Splenectomy of the pigeon abrogates the antibody response and the birds die soon after pneumococcus infection.

The splenic anlage develops from the dorsal mesogastrium, ventral to the notochord and its ablation at the lower thoracal level abrogates splenic development, suggesting a notochordal inductive effect. Haematopoietic colonization of the splenic anlage begins about 6.5 days of incubation, contemporaneous with that of the thymus. It is also worth mentioning that in the spleen not only the cells of the haematopoietic compartment but also other resident cells, like the ellipsoid-associated cells (phagocytic) and supporting cells of the ellipsoid (capable of collagen production) are of blood-borne origin (Nagy *et al.*, 2005).

Several peripheral, or secondary, lymphoid tissues can be distinguished in the chicken, most notably the spleen and mucosa-associated tissues such as the eye-associated lymphoid tissue (Harderian gland and in the conjunctiva of the lower eyelid), nasal-, bronchus-, genital- and gut-associated lymphoid tissues (oesophageal tonsils, pyloric tonsils, Peyer's patches (PP), caecal tonsils (CT) and Meckel's diverticulum) and the skin- and pineal-associated lymphoid tissues.

Secondary lymphoid organs, unlike the thymus and bursa, are not sites for antigen-independent differentiation and lymphocyte proliferation. Most organized lymphoid tissues begin to develop independently of antigen stimulation, since they are found in predilected sites before hatching. However, further maturation of the lymphoid tissues is antigen driven, as can be demonstrated using germ-free chickens (Hedge *et al.*, 1982).

Lymphoid organs are highly compartmentalized. Each anatomical compartment has its own typical arrangement of lymphoid and non-lymphoid cells, with separate B- and T-dependent areas. The different types of non-lymphoid cells create the specific microenvironment in each of the compartments. Separate areas exist where antigen presentation to T cells by non-lymphoid cells occurs, T cells interact with B cells and immunoglobulin (Ig) production takes place. It is therefore important to realise that *in vitro* studies generate information on the possible interactions between cells, though some of these interactions are prevented *in vivo* due to compartmentalization. An appreciation of the detailed structure of an organ is essential for a complete understanding of its functions.

The development of secondary lymphoid organs begins when T and B cells intermingle and then separate into distinct T and B cells areas, followed by the induction of GC in T cell areas. With separation into T and B cell areas, non-lymphoid cell populations develop at characteristic sites.

Non-lymphoid cells can be classified into two groups. The first consists of epithelial cells, endothelial cells and connective tissue cells including the reticular and follicular dendritic cells (FDC). Endothelial cells form the inner lining of arteries, veins and capillaries and lymphocytes cross this endothelium to enter lymphoid tissue. Specialized vessels with high endothelial cells that bulge into the lumen (HEV) facilitate entry of lymphocytes using specific adhesion molecules which interact with tissue-specific lymphocytic homing receptors. These endothelial cells have a large number of mitochondria and ribosomes suggesting an increased state of activity which may be related to their functions, transporting both cells and antigens. Reticular cells and their extracellular matrix form the basic framework of lymphoid tissues, the reticulum. Little is known about reticular cells, which could have direct involvement in the regulation of immune functions by guiding the migration and anchorage of lymphocytes to their respective compartments. Reticular cells contain glycoproteins, for which many lymphocytes express adhesion molecules.

The second group is constituted by macrophages and antigen-presenting dendritic cells (DC), which belong to the mononuclear phagocyte system (Van Furth, 1970; Van Furth *et al.*, 1972). This system includes all cells derived from monoblasts in the bone marrow. These cells are described in Chapter 9.

THE GC OF PERIPHERAL LYMPHOID ORGANS

Histologically, the GC is defined by aggregates of blast cells which, after antigenic stimulation, occur in defined areas within peripheral tissues. In chickens, two types of GC have been described,

fully and partially encapsulated. Although the cell populations of fully and partially encapsulated GC do not differ cytologically, there is speculation that these represent functionally different structures (Oláh and Glick, 1979). Alternatively, they could represent different stages of GC development (Jeurissen and Janse, 1994). Mature GC are surrounded by a capsule of connective tissue and completely lack blood vessels. They are located close to T cell areas adjacent to arteries and arterioles.

In the spleen, the induction site of GC seems to be directed by clusters of stromal cells, recognized by the monoclonal antibody (mAb) CVI-ChNL-74.3, situated near the arteries and arterioles in the unstimulated spleen. During a humoral response, newly developing GC, which consist of proliferating bromodeoxyuridine (BrdU)-incorporating B cells, are located adjacent to clusters of -74.3$^+$ cells. During development B cells grow around these clusters until they completely surround them. Cells in the centre of the GC do not incorporate BrdU, hence, they are not mitotically active and resemble centrocytes; whereas the outer ring of proliferating cells ressemble centroblasts (Fig. 2.1(a)). Although morphologically, a light zone of centrocytes and dark zone of centroblasts, as described for mammalian GC, can be less easily discriminated in the chicken, functional homology seems likely (Oláh and Glick, 1979; Jeurissen and Janse, 1994; Yasuda *et al*., 1998). In mature GC, -74.3$^+$ cells can trap immune complexes on the surface and are called FDC; these trapped immune complexes are important in memory formation. Specific antibodies or antibody-containing cells are detected in serum and spleen, respectively, before the formation of GC. Therefore, the site of GC formation is not related to that of the simultaneously induced antigen-specific plasma cells, as reported for the mouse spleen (Jeurissen and Janse, 1994; Yasuda *et al*., 2003).

A mature GC consists of proliferating B cells, few T cells expressing CD3, CD4 and the $\alpha\beta$1-T cell receptor (TCR) and FDC (-74.3$^+$ and S-100$^+$ cells) but only scattered CD8$^+$ cells (Fig. 2.1(b)). Arakawa *et al*. (1996) demonstrated that the antigen-activated B cells of the spleen migrate into the GC, where they expand, as proposed for human GC development (Küppers *et al*., 1993). These workers estimated clonal complexity in each splenic GC to be of the order of 6–12, suggesting that 6–12 B cell clones are expanding with each producing a different antibody.

Contradictory results concerning Ig expression in GC have been reported. Mast and Goddeeris (1998a) described three B cell populations, based on expression of CD57 and the B cell marker, chB6 (also referred to as the Bu-1 antigen). The three populations were: resting B cells, plasma cells and GC B cells. B cells in the GC were CD57$^+$ chB6$^+$ but either negative, or weakly positive, for Ig. They suggested that the activation of B cells induces expression of CD57 but downregulates Ig.

In contrast, Jeurissen *et al*. (1988a) described follicle B cells expressing surface (s) IgM but not sIgY. Yasuda *et al*. (2003) were able to isolate individual GC from the spleen and analyze GC cell populations at an individual level. They described considerable differences in sIgM and sIgY expression between GC. Ig-expressing cells were found in the centre of the GC but not in the outer rim where proliferating B cells occur. The relative proportions of sIgM$^+$ and sIgY$^+$ cells changed after intravenous (i.v.) inoculation of dinitrophenol–keyhole limpet haemocyanin (DNP–KLH) with the highest proportion of sIgM$^+$ cells being detected on day 7. The proportion of sIgY$^+$ cells increased during the first 14 days, but, as observed for sIgM$^+$ cells, a large individual variation was evident. By contrast, the proportion of CD3$^+$ cells remained relatively constant in each GC. Hitherto, double-staining studies have not elucidated the nature of the isolated sIg$^+$ cells, i.e. the FDC and B cells.

THYMUS

Anatomy and Histological Organization

The avian thymus lies parallel to the vagus nerve and internal jugular veins (Hodges, 1974). On each side of the neck there are 7–8 separate lobes, extending from the third cervical vertebra to

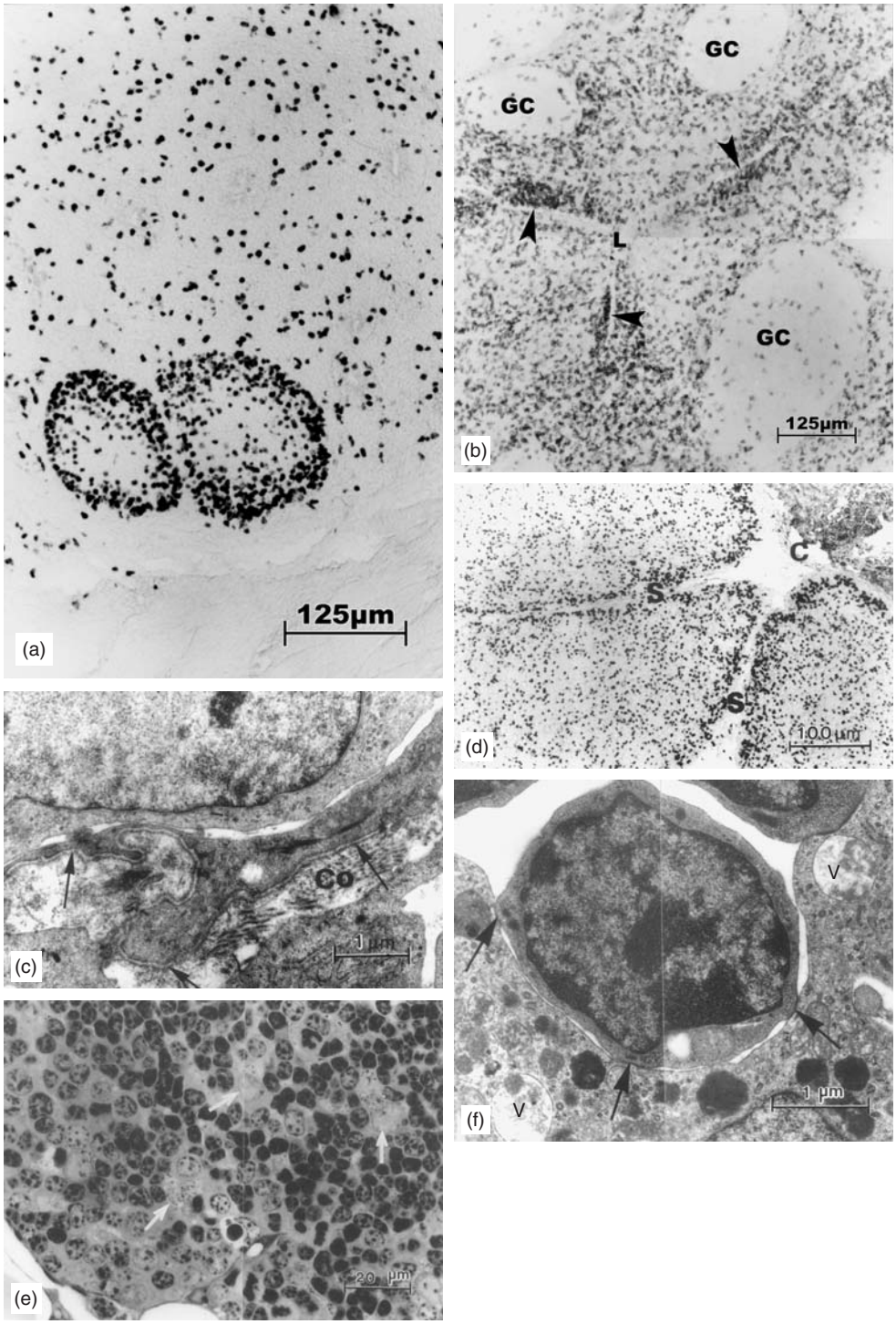

FIGURE 2.1 Proliferating cells in the germinal centres (GC) and thymic cortex. (a) BrdU immunocytochemistry showing the proliferating zone is in the periphery of the chicken's GC where FDC are absent. FDC are localized in the centre where B cell proliferation is low. (An electron micrograph of a FDC is shown in the Fig. 2.9(e)). (b) CD8 immunohistochemistry of GC reveals scattered $CD8^+$ cells, while the lymphoepithelial tissue (surface epithelium of the caecal tonsil) is heavily loaded with CD8 positive cells (arrow), L: lumen. (c) Basal lamina (arrow) covers the surface epithelial cell and separates the thymic parenchyma from the connective tissue capsule. Co: capsular collagen fibres. (d) BrdU immunocytochemistry shows proliferating cells accumulating under the subcapsular zone. Cell proliferation occurs over the entire cortex. C: capsule; S: septa. (e) The densely packed cortical thymocytes result in basophilic staining. Large, pale nuclei belong to the ERC (arrow). (f) Attachment spots between the cortical thymocytes and the ERC (arrow). Granules show different electron density and the membrane bound vesicles (V) contain a fine flocculated substance.

the upper thoracal segments (Kendall, 1980). Each lobe is encapsulated with a fine fibrous connective tissue capsule and embedded into adipose tissue (see Plate 1.1(a)). From the capsule, septae invade the thymic parenchyma and incompletely divide the lobe into lobules. The button- or bean-shaped thymic lobes reach a maximum size of 6–12 mm in diameter by 3–4 months of age, before physiological involution begins (Ciriaco *et al.*, 2003). The thymic parenchyma of each lobe consists of a centrally located uniform medulla surrounded by a lobulated cortex. Septae end at the cortico-medullary border leaving the medulla undivided. Arteries travel in the septum entering the thymic parenchyma at the end. Beside the arteries, capillaries, veins and efferent lymphatics are also found. Septae have a rich cellular composition consisting of fibroblasts, plasma cells, lymphocytes and, occasionally, a few granulocytes. The surface of the lobules is isolated from the capsule and septae by a basal lamina (BL; Fig. 2.1(c)). The connective tissue of the thymic capsule and septae develop from the cells of the cranial neural crest. Lack, or impaired migration, of neural crest cells to the third and fourth brachial arches results in a thymic condition like Di George syndrome in man and nude mice.

During embryonic development the thymic mass gradually increases with colonization of haematopoietic stem cells (Le Douarin and Jotereau, 1975) and the rapid increase, a few days before hatching, results in the appearance of the medulla. Histological differentiation continues after hatching. Blood-borne haematopoietic cells invade the epithelial anlage of the thymus and immature and proliferating T lymphocytes are present in the subcapsular zone (Fig. 2.1(d)), from where they migrate towards the medulla during T cell maturation. Immunologically competent cells leave the thymus via medullary post-capillaries. The formation of the thymus and the invasion of the connective tissue septae result in cortical lobulation, which coincides with the emigration of T cells from the thymus. Thymic reticulum develops from the endoderm of the third and fourth branchial pouches.

Thymic Cortex

Pale-stained, fine cytokeratin meshes of the epithelial reticular cells (ERC) in the cortex are densely packed with thymocytes, contributing to cortical basophilia (Fig. 2.1(e)). Moderate numbers of macrophages also occur in the network of cortical epithelial cells, and large- and medium-sized lymphocytes are situated under the surface epithelial cells, which are covered by a BL towards the capsule and septae (Fig. 2.1(c)). Many of these lymphoblasts are in S phase of the cell cycle, suggesting that the subcapsular zone of the cortex is the major site of proliferation (Fig. 2.1(d)). During T cell maturation the cells migrate towards the cortico-medullary border, where macrophages and thymic DC sentinel and negatively select the thymocytes, before these enter the medulla and circulation via the medullary post-capillaries.

The fine cytokeratin network of the cortex is formed by the cortical ERC. Two types of ERC can be identified, though it is difficult to distinguish between them cytologically. One is located on the surface of the lobe and contains few, or no, cytoplasmic granules but expresses more cytokeratin than cortical ERC. Possibly, its major function is to isolate the cortical parenchyma from the mesenchyme, which surrounds the thymic lobe and participates in the cortical blood-thymus barrier (Fig. 2.1(c)). These cells are connected by desmosomes to one another and to the cortical ERC. The latter cells have large, irregular-shaped nuclei with evenly dispersed chromatin substance (Fig. 2.1(e)). The cytoplasm is rich in mitochondria and loaded with granules, which exhibit variations in shape and electron density. The ends of the cytoplasmic processes contain moderately developed smooth- and rough-surfaced endoplasmic reticulum. Keratin positive tonofibrils are occasionally observed. Their relationship to the cortical thymocytes is a remarkable cytological phenomenon. Between each epithelial cell and thymocyte several adhesion points occur, indicating that cell-to-cell contact may be significant in T cell maturation (Fig. 2.1(f)), and it suggests a nursing function for ERC (Reike *et al.*, 1995). Several granules contain very loose, low-density material suggesting partial release of their granular

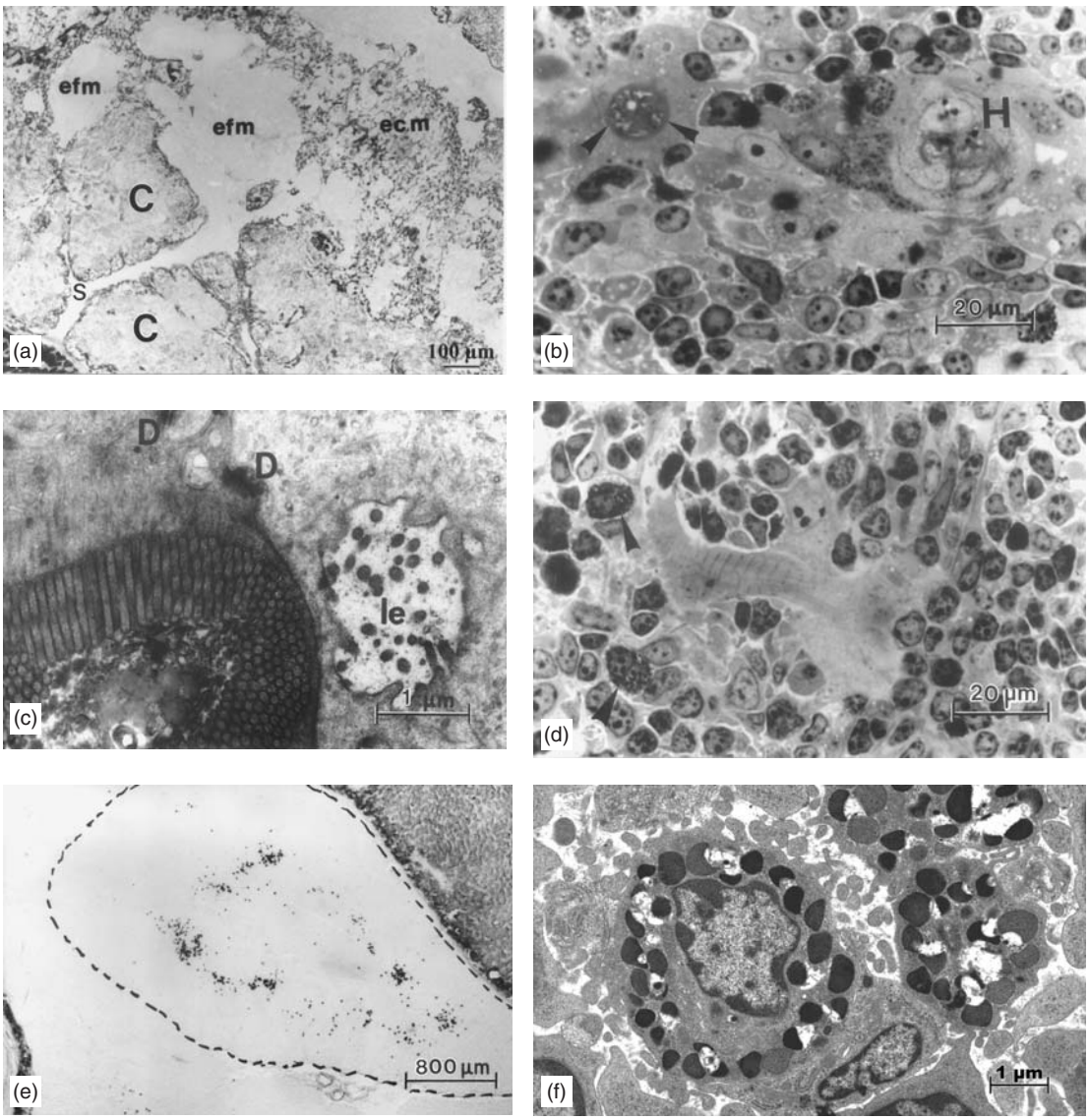

FIGURE 2.2 Detailed structure of the thymus. (a) Pan-cytokeratin immunostaining shows very fine network in the cortex (C), while the space of the septae (S) is continuous with the epithelial-free medullary (efm) region. The epithelium containing medullary (ecm) region has a rough cytokeratin network. (b) Hassall's body (H) shows concentric appearance, while on the upper left corner of the picture there is a cyst with brush border (dark ring on the edge of the cyst, arrow). (c) Intraepithelial (Ie) and interepithelial cyst, which is lined by microvilli. D: desmosome. (d) Skeletal muscle cell (myoid) in the medulla shows regular I and A bands. Peroxidase positive cells (arrow). (e) At the cortico-medullary border there is a peroxidase positive cell barrier. The cells form small groups, suggesting their clonal origin. (f) Electron micrograph of the peroxidase positive cell. The irregular-shaped nucleus is surrounded by granules. Many of them show granulolysis.

contents (Fig. 2.1(f)) giving the appearance of vesicles. The proportions of numbers of granules and vesicles changes from cell to cell, which may be explained as different functional stages. The large granules are, surprisingly, not membrane-bound, but sharply separated from the cytoplasm. However, when the granular content is released membrane-bound vesicles remain. This type of cell occurs exclusively in the cortex, although at the subcapsular zones there also may be a BL.

Thymic Medulla

The large number of highly variable epithelial-like cells and smaller number of thymocytes in the medulla result in less basophilic staining. The two medullary regions can be distinguished in sections stained with anti-cytokeratin (Fig. 2.2(a)). The majority of epithelial-like cells with low electron density form clusters that possibly occur in the epithelial-free regions. These cells are identified histologically as epithelial cells, even though they do not express cytokeratin filaments. Their shape is cuboidal rather than stellate and frequently they produce dense granules reminiscent of the dense-core vesicles of adrenal medullary cells. This could be indirect evidence for the contribution of neural crest cells to the medullary region. Epithelial cells can be classified into several types but it is not known whether they represent actual cell types or stages of differentiation. Their functional significance is not known but an endocrine function has been suggested (Isler, 1976; Glick, 1980; Audhyne *et al*., 1986).

Medullary epithelial cells can form intra- and inter-epithelial cysts (Fig. 2.2(b) and (c)). In the former few irregular microvilli occur, while the latter produce a proper brush-border of the microvilli with a regular diameter and length, reminiscent of intestinal epithelial cells. The lumen of these cysts is frequently filled with an electron-dense substance, indicating that these cyst-forming epithelial cells are secretory (Fig. 2.2(b) and (c)). Medullary epithelial cells have strong desmosomal connections, since they have large surface contact to which tonofibrils (keratin-intermediate filaments) are attached.

The classical histological structure in the thymic medulla is Hassall's corpuscle, an epithelial cell aggregation (Fig. 2.2(b)). In chickens these are small, poorly developed structures, unlike their human counterparts. Their function remains an enigma, although it has been proposed that the turnover of the epithelial cells results in Hassall's corpuscles, since the centre frequently shows keratinization like the skin epidermis. Others regard it as a place of thymocyte degeneration; though thymocytes are rarely observed in them unlike in neutrophils (mammals) or heterophils (birds). These granulocytes are probably scavenger cells, eliminating the Hassall's corpuscles. Over the past quarter century several authors have reported the production of biologically active substances, such as alpha-naphthylesterase and leucinaminopeptidase (D'Anna *et al*., 1981), interleukin (IL)-7 (He *et al*., 1995), transforming growth factor alpha (Le *et al*., 1991), CD30 ligand (Romagnani *et al*., 1998), an IL-7 like cytokine called thymic stromal lymphopoietin (TSLP; Watanabe *et al*., 2005; Liu, 2006). TSLP is produced by the Hassall's corpuscles in the human thymus and TSLP receptors are preferentially expressed by immature medullary myeloid DC. TSLP activates myeloid DC which generate regulatory T cells in the thymic medulla (Liu, 2006). The high affinity receptor for TSLP and the IL-7 receptor (IL-7R) share the IL-7Rα chain. The chicken IL-7Rα chain is expressed on many cortical thymocytes (less so towards the capsule) and some medullary cells suggesting a T cell maturation-dependent expression similar to the mammalian thymus (Sudo *et al*., 1993; L. Vervelde, unpublished observations).

Skeletal muscle cells are a common feature in the thymic medulla (Fig. 2.2(d)). Some are round or ovoid in shape and striated myofibrils encircle the nucleus. Others are elongated and their ends appear Y-shaped. There are no signs of innervation to these skeletal muscles.

Thymic Cortico-Medullary Border

The cortico-medullary border contains a DC barrier which expresses the major histocompatibility complex (MHC) class II antigen and may play a role in the negative selection of T cells (Guillemot *et al*., 1984). In addition to DC, a large number of peroxidase-positive cells also occur at the cortico-medullary border and these may also contribute to the negative selection of the T cells (Oláh *et al*., 1991). These endogenous peroxidase-positive cells generally form groups and between them single cells occur (Fig. 2.2(e)). Their irregular-shaped granules show granulolysis and the cell surface is highly ruffled (Fig. 2.2(f)). Peroxidase-positive cells do not have segmented nuclei and the ultrastructure of their cytoplasmic granules is not comparable

to that of eosinophilic granulocytes. Granulocyte-specific mAb (Gr1 and Gr2) do not recognize these thymic peroxidase-positive cells.

BURSA OF FABRICIUS

Anatomy and Histology of the Bursa of Fabricius

The chicken bursa of Fabricius has the shape and size of a chestnut and is located between the cloaca and the sacrum (Plate 1.1). A slot-like bursal duct provides a continuous and free communication between the proctodeum and the bursal lumen. As a diverticulum of the cloaca, the bursa is lined by a cylindrical epithelium thought to be of entodermal origin. The bursa reaches its maximum size at 8–10 weeks of age then, like the thymus, it undergoes involution. By 6–7 months most bursae are heavily involuted (Ciriaco *et al.*, 2003).

The bursa is surrounded by a thick, smooth muscle layer like other hollow organs. Studies generally neglect this muscle coat, and its contractility is not considered in bursal function. During muscle contraction compression of the follicles can promote the flow of cells within the medulla and contribute to the emptying of lymphatics situated in the axis of the folds. Into the bursal lumen 15–20 longitudinal folds emerge, resulting in a slit-like space. During muscle contraction the surfaces of the folds come into contact with one another, so the bursal lumen is almost a virtual space. Inside each fold follicles are organized into two layers separated by axial structures (arteries, veins, lymphatics and connective tissues) (Fig. 2.3). Consequently, follicles are in contact with both blood and lymphatic vessels as well as the bursal lumen. In one of the ventral folds peripheral (secondary) lymphoid tissue is formed.

Bursal Surface Epithelium

The surface epithelium of each fold consists of interfollicular epithelium (IFE) and follicle-associated epithelium (FAE; Bockman and Cooper, 1973) that form about 90 and 10% of the surface, respectively (Fig. 2.4(a); Oláh and Glick, 1978a). IFE is columnar and produces a mucin-like

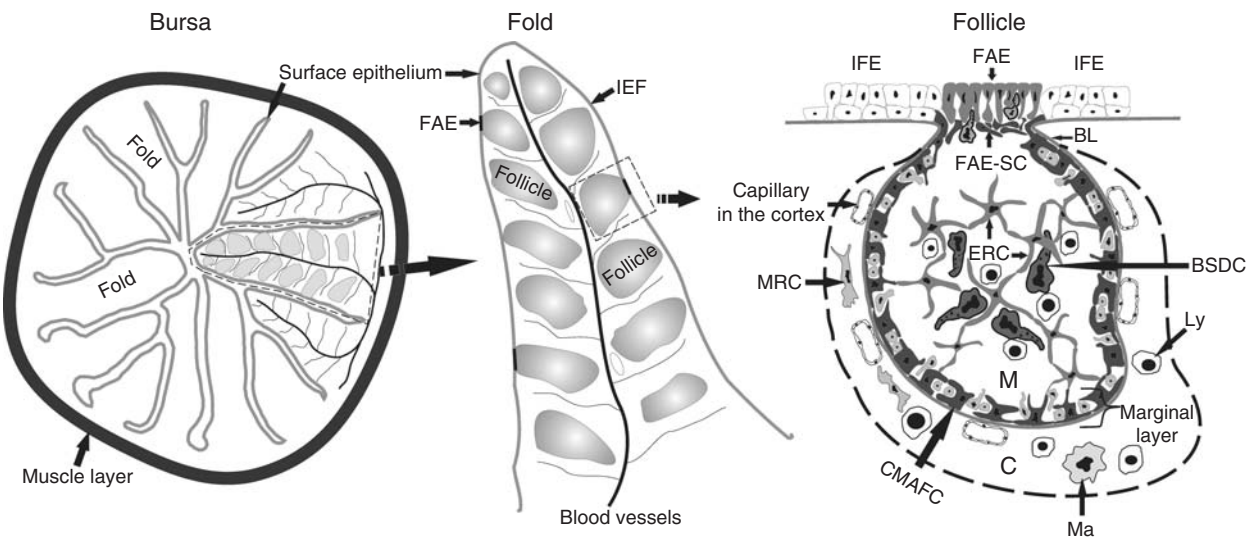

FIGURE 2.3 The structure of the bursa of Fabricius. A schematic drawing of the histological organization of the bursa of Fabricius. CMAFC: cortico-medullary arch-forming cell; FAE-SC: follicle-associated epithelium supportive cell; ERC: epithelial reticular cell; BSDC: bursal secretory dendritic cell; IFE: interfollicular epithelium; BL: basal lamina; Ly: lymphocyte; Ma: macrophage; M: medulla; C: cortex; MRC: mesenchymal reticular cell.

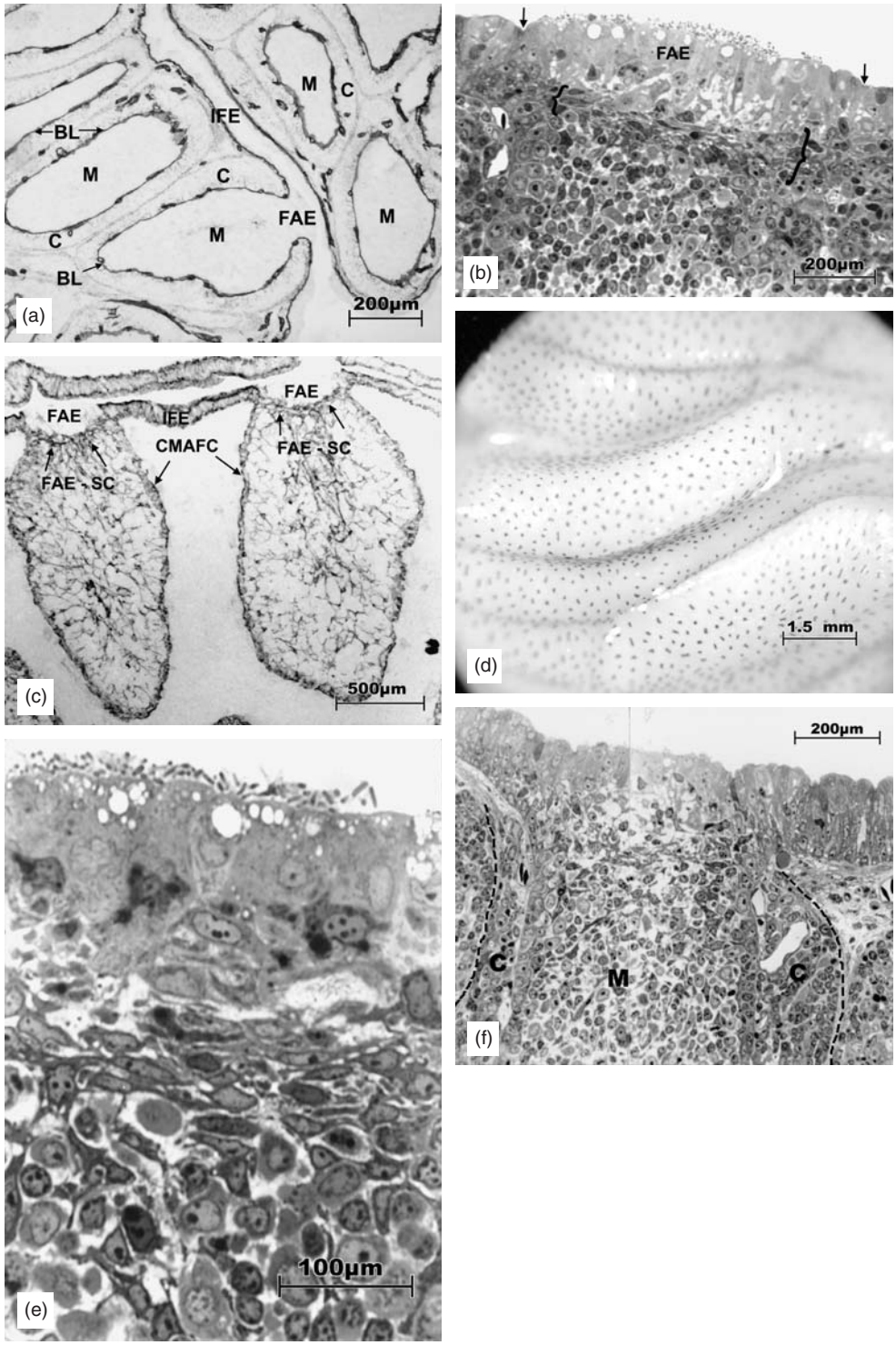

FIGURE 2.4 Detailed structure of the bursa of Fabricius. (a) Anti-laminin immunostaining indicates that the BL under the interfollicular epithelium (IFE) continues at the cortico-medullary border (arrow). Follicle-associated epithelium (FAE) medulla (M) and cortex (C). (b) The FAE sits on top of the follicle and is separated from the IFE by a shallow trough (arrows). A large number of bacteria are attached to the FAE. The medulla is isolated from the FAE by the FAE-supportive epithelial cells indicated by { }. (c) Anti-cytokeratin staining identifies IFE, FAE supportive cells and ERC. The CMAFC have a double-layered, compartmentalized pattern. (d) A carbon particle suspension dropped onto the anal lips is sucked up into the bursal lumen. The carbon is absorbed by the FAE, allowing counting of the number of follicles. Each black dot covers one FAE. (e) In the FAE three macrophage-like cells can be seen. Under the follicle associated epithelium (FAE) basal lamina is absent. There are no lymphocytes in the FAE. Bacteria are above the FAE. (f) The FAE is closely associated with the medulla. The basal lamina (BL) of the IFE goes on between the cortex (C) and medulla (M). The collagen capsule of the follicle is shown by dashed line.

substance, which is released into the bursal lumen lubricating the surface of the folds. The columnar epithelium is pseudostratified, since there is a layer of cuboidal basal cells with densely stained cytoplasm. The function is not known. Perhaps the cuboidal cells are predestined for the IFE, since the surface epithelium does not proliferate and no epithelial stem cell has been identified. The IFE rests on a BL, continuous with the cortico-medullary border separating the medulla from the cortex (Figs 2.3 and 2.4(a)). It is generally accepted that the medulla is of entodermal origin, and so has similar origins to the thymic medulla.

FAE covers the bursal fold above the follicles and provides a direct connection between the follicular medulla and the bursal lumen (Figs 2.3 and 2.4(b)). A special smooth-surfaced vesicular system in the apical zone of the FAE cells (function not known) may contribute to the bidirectional transport by these cells (as proposed by Bockman and Cooper, 1973). Antigen or particles can gain access to the medulla from the bursal lumen (Schaffner *et al*., 1974; Oláh and Glick, 1978a; Naukkarinen and Sorvari, 1984) and in the opposite direction products of bursal secretory dendritic cells (BSDC) are also taken up by the FAE (Nagy *et al*., 2001). During late embryonic life such products appear in large quantities in the medulla and enter the intercellular space of the FAE. Cells of the FAE take up and possibly secrete these products into the bursal lumen (Nagy *et al*., 2004a). FAE express non-specific esterase, like the macrophages, which suggests that their origin is mesenchymal. Around 15–16 embryonic incubation days (EID) mesenchymal cells invade the surface epithelium and transform to become FAE cells (Lupetti *et al*., 1990). This finding has been confirmed with guinea fowl using a DC-specific mAb (Nagy *et al*., 2001, 2004a). Anti-pan-cytokeratin staining indicates the absence of keratin-intermediate filaments in the FAE, although each epithelial medullary component expresses keratin (Fig. 2.4(c)).

The FAE is an attachment point between the follicle and the surface epithelium, so the number of FAE attachments is identical to the number of follicles (Fig. 2.4(e)). Under each FAE the BL is absent and (unusual for an epithelium) replaced by 2–3 layers of squamous epithelial cells, called FAE-supportive cells (Fig. 2.4(b) and (c)). The FAE cells are connected to the FAE-supportive cells by desmosomes. The flat FAE-supportive cells together with the cortico-medullary arch-forming epithelial cells envelop the medulla separating it from the FAE and cortex, respectively (Fig. 2.4(b)). The FAE is devoid of lymphocytes but is penetrated by macrophage-like cells (Fig. 2.4(e)), although these cells do not stain with typical monocyte/macrophage markers and may represent senescent BSDC, that have migrated from the medulla (Oláh and Glick, 1992). FAE cells are functionally identical to the M cells of the gut-associated lymphoid tissues (GALT). However, classical M cells are capable of stimulating lymphocytes so the absence of any lymphocytes in the FAE raises questions about these FAE cells being M cells.

Soluble substances dropped onto the cloacal lips are rapidly sucked into the bursal lumen (Schaffner *et al*., 1974; Oláh and Glick, 1978a; Romppanen *et al*., 1983; Naukkarinen and Sorvari, 1984). The mechanism of this suction is not known, although it could be due to negative pressure within the bursal lumen. The bursal duct is flat, not circular, and therefore generally closed. Absorption of the air from the bursal lumen could result in a negative pressure with contraction of the cloacal sphincter opening up the bursal duct. By these mechanism tracers, bacteria and viruses can gain access into the bursal lumen, to be absorbed by the FAE (Fig. 2.4(d) and (e)). Colloidal carbon is taken up by the FAE providing a means to count the number of follicles, estimated to be 20,000/bursa. During folliculogenesis there is no cell migration between follicles therefore, assuming a minimum one B cell precursor per follicle, this means 20,000 pre-B cells are required for bursal colonization (Oláh and Glick, 1978a).

Bursal Follicle

Each bursal follicle consists of a medulla and cortex with a closely associated (both structurally and functionally), but not integral, FAE (Bockman and Cooper, 1973). During the bursal ontogeny the medullary anlage emerges on the 11–12 EID and is soon followed by formation of the FAE (14–15 EID) (Bockman and Cooper, 1973) with the first cortical cells appearing around hatching

(Oláh *et al*., 1986; Pike *et al*., 2004; Ratcliffe, 2006). The cortex is fully developed by 2 weeks after hatching.

Bursal folds are filled with follicles, which are flattened, oval-shaped, ~0.2–0.4 mm in diameter. A collagen-rich capsule surrounds each follicle, which represents the structural, functional and pathological bursal unit (Fig. 2.4(f)). Each follicle has its own blood supply independent of neighbouring follicles. Small pre-capillaries branch from the main artery in the fold and cross the follicular cortex creating a dense capillary network at the cortico-medullary border. Blood vessels never enter the medulla. A blood/bursa barrier may exist in the medulla, though not in the cortex. Early studies concerning phagocytic capability in the bursa support the existence of a blood/bursa barrier, as no phagocytosed substances occur in the bursa.

The cortico-medullary BL provides a complete separation of the medulla from the cortex (Fig. 2.4(a)), because these two regions are different histologically, ontogenically and possibly functionally. Supporting cells in the medulla and the cortex are of epithelial and mesenchymal origin, respectively.

Bursal Medulla

The medulla is a classical lymphoepithelial tissue like the thymus. At EID 10–13, blood-borne precursors of BSDC entering the surface epithelium induce bud formation towards the mesenchyme. The epithelial cells of the bud and the precursors of the BSDC form a transitory dendro-epithelial tissue (Nagy *et al*., 2004b), which is capable of receiving B cell precursors (Le Douarin *et al*., 1975). Thus, haematopoietic colonization of the bursal follicles occurs in two stages:

1. Formation of dendro-epithelial tissue, which can be inhibited by embryonic testosterone treatment (chemical bursectomy; Oláh *et al*., 1986).
2. Colonization of dendro-epithelial tissue by pre-B cells (Le Douarin *et al*., 1975).

The cellular composition of the medulla is relatively simple and stable. It consists of:

1. Epithelial cells, which may originate in the cloacal diverticulum.
2. Blood-borne haematopoietic cells, including DC, lymphoid cells and macrophages with a few plasma cells in the involuting bursa.

Bursal Epithelial Cells

Epithelial cells can be classified according to their location and cellular structure. The superficial epithelial cells form a layer on the surface of the medulla. At the cortico-medullary border these cells form arches on the medullary side and are covered by a BL on the cortical side. Under the FAE the arch-forming cells become flat and serve as supportive cells for the FAE, replacing the BL. Hence, the interior of the medulla is separated from the cortex and FAE. The cortico-medullary arch-forming cells enclose lymphoblast-like cells, which express neither B cell (e.g. chB6) nor BSDC (e.g. vimentin, IgY and -74.3) antigens. Ramm *et al*. (1991) suggested that the cells at the cortico-medullary junction are resistant to infectious bursal disease virus (IBDV) and represent a macrophage subpopulation. Anti-cytokeratin staining reveals double-layered cortico-medullary epithelial cells, which form compartments for the blast-like cells (Fig. 2.4(c)). Delta-notch signalling in the cortico-medullary border also suggests that the function of the arch-forming cells differs from that of the medullary ERC (Morimura *et al*., 2001). These cortico-medullary arch-forming cells could have some kind of nursing or regulatory function. If the blast-like cell population is exhausted after IBDV infection or testosterone treatment, regeneration of the follicle, but not the entire bursa, is handicapped.

The cytological structure of the cortico-medullary and FAE-supportive epithelial cells is quite different. The former are rich in cytoplasmic organelles and their cytoskeletal keratin filaments outline the arch shape of the cells, while FAE-supportive cells form 2–3 squamous cell layers that are rich in keratin with few organelles.

In the medullary interior the epithelial cells are stellate and form a three-dimensional network for B cells and BSDC. Epithelial cells are connected by desmosomes to one another and to the cells of the FAE-supportive and arch-forming cells. Their cytological structure is comparable with that of the FAE-supportive cells, namely rich in keratin and with poorly developed cytoplasmic organelles, suggesting a supportive rather than secretory function (Fig. 2.4(c)).

Before hatching the bursal epithelial cells co-express keratin and vimentin-intermediate filaments (Oláh *et al*., 1992) but shortly after hatching vimentin expression ceases. In acute bursal involution, induced by B cell-depleting agents or IBDV, vimentin expression re-emerges in the epithelial cells, suggesting that B cell depletion results in de-differentiation of epithelial cells, i.e. a return to the embryonic state. Co-expression of keratin and vimentin-intermediate filaments by the epithelial cells may be prerequisite for the bursal follicular regeneration, i.e. for colonization of the follicles. Natural involution begins around 12 weeks of age, preceding sexual maturation, but without vimentin re-expression in ERC.

Bursal Secretory Dendritic Cells

This cell, first identified by Oláh and Glick (1978b), is only found in the medulla. Cyclophosphamide eliminates B cells from both the medulla and cortex, but does not affect BSDC. BSDC have two cellular processes, which are different in size and cytological structure. The smaller one contains few rough-surfaced endoplasmic reticulum cisternae, free ribosomes and a large number of vimentin-intermediate filaments. The larger cell process contains many membrane-bound electron-dense granules around a well-developed Golgi region, or it lines up along the cell membrane (Fig. 2.5(a)). The arrangement of the cytoplasmic organelles in the cell gives the mature BSDC a polarized appearance (Fig. 2.5(a)). The mature BSDC is highly elongated in shape and a nuclear chromatin structure similar to that of the lymphocyte (Fig. 2.5(a)). Granular contents are released and attach to the outer surface of the cell membrane (Fig. 2.5(a) and (b); Oláh and Glick, 1987). This substance may become detached from the cell membrane and solubilized in the medulla (Fig. 2.5(b)). The soluble form can cross the BL of the cortico-medullary border and appear in the cortex or enter the circulation. Cell adhesion sites can be observed between the BSDC and the lymphocytes (Nagy *et al*., 2004b).

The biochemical composition of the granules is not known, but the mAb CVI-ChNL-74.3 recognizes intracellular antigens, possibly granules (Jeurissen *et al*., 1994). IBDV infection eliminates the -74.3^{+} staining suggesting BSDC are a target for IBDV. It is important to point out that the antigen recognized by mAb -74.3 has not been characterized, but double staining with vimentin clearly indicates co-localization. Thus, these two mAb are convenient tools for monitoring the condition of BSDC. sIgY appears on BSDC just before hatching and persists throughout life, so anti-IgY can also be used for their identification. IBDV infection eliminates not only the B cells but also sIgY, confirming the involvement of BSDC in the IBDV pathogenesis. After hatching the number of BSDC increase until 4–6 weeks of age. At hatching 5–8 vimentin positive BSDC per follicle are present increasing to 65–70 by 4–6 weeks of age.

The life cycle of the BSDC can be summarized as follows: precursor cells in the cortico-medullary arches are capable of proliferation; with the opening of the arches precursors enter the interior of the medulla where their proliferation ceases but differentiation begins; immature BSDC have copious cytoplasm with a blast-like nucleus and few granules, and express intermediate filament; the mature BSDC is a highly elongated, polarized cell, which releases granular contents; the senescent BSDC is phagocytic and appears as a tingible-body macrophage. The cell loses the -74.3 antigen, sIgY and expression of vimentin-intermediate filaments but the cells maintain their MHC class II antigen (Fig. 2.5(c)); senescent cells enter the FAE and are eliminated into the bursal lumen. The life cycle is accelerated by IBDV infection, together with FAE the BSDC can leave the medulla through the BL of the cortico-medullary border.

The cortico-medullary arch-forming cells promote the proliferation of BSDC precursors, and B cells which are nearby (Fig. 2.5(d)), but inhibit their differentiation, suggesting a regulatory function, while the ERC allow them to differentiate (Oláh *et al*., 2002a).

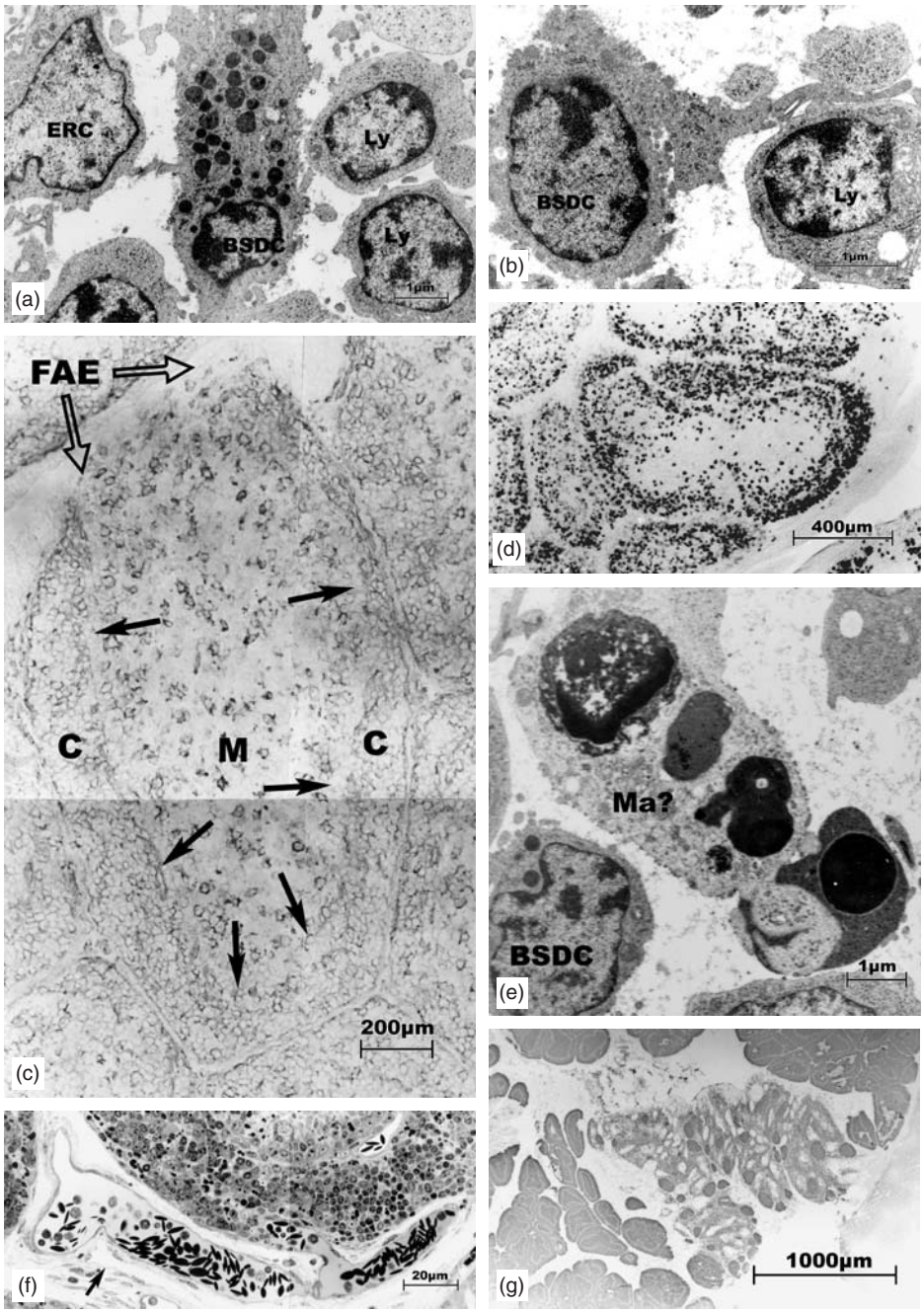

FIGURE 2.5 Details of cells in the bursa of Fabricius. (a) Electron micrograph of the medulla showing the mature dendritic cell has an excentrically located nucleus with a large cell process that is loaded with electron-dense granules. The moderately ruffled cell surface is covered by a fine filamentuous substance. Ly: lymphocyte. (b) BSDC sectioned through the nucleus without cell processes. The cell membrane is heavily loaded with a fine filamentuous substance. Compare the cell surface of the BSDC with that of the lymphocyte. (c) MHC class II immunostaining. B cells in the cortex (C) express anti-Ia antigen, unlike the medullary ones. The BSDC of the medulla (M) are heavily, or moderately, positive. Few Ia positive cells occur in the FAE. There is an anti-Ia negative band in the cortico-medullary junction, which corresponds to the arches (arrows). (d) BrdU immunocytochemistry indicates that the major proliferating regions in the bursal follicle are in close association of the marginal zone of the medulla. (e) The surface membrane of a macrophage-like cells is covered with a substance comparable with that of the BSDC. (f) A tributary of a post-capillary in the connective tissue of the fold. On the left side below the post-capillary, a lymphatic appears without cells (arrow). (g) Between two bursal folds the peripheral lymphoid tissue consists of germinal centres and dense lymphoid tissue as shown by immunostaining for the chB6 (Bu-1) antigen.

Bursal Macrophages

In histological sections macrophages with phagocytic substances are easily identified in both the cortex and medulla; however, no positive cells were recognized using mAb against monocyte/macrophage markers, such as -74.2, -68.1, -68.2 and KUL-01. This suggests that either the bursal follicle does not have macrophages or its macrophage population is unique. After IBDV infection large cells loaded with cellular debris and virus particles appear first in the medulla and later in the cortex, while BSDC cannot be identified using any cell marker. It is worth mentioning, that the electron micrographs show a surface substance on several macrophages, comparable to that on BSDC (Fig. 2.5(e)). This finding, together with the MHC class II^+ macrophage-like cells in the FAE indicates that the medullary macrophages are senescent BSDC (Oláh and Glick, 1992). In other words, the terminal stage (or maturation) of the BSDC could be a bursa-specific macrophage capable of penetrating FAE-supportive cells, entering the FAE and being eliminated into the bursal lumen.

Bursal Lymphocytes

At least 98% of the bursal lymphocytes are B cells. Scattered T cells occur in the cortex but few of them enter the medulla. B cells proliferate in both the cortex and medulla. After 8–10 weeks of age the number of lymphocytes in the interior of the medulla decreases indicating the initiation of the bursal involution, a process, completed by 6–7 months of age. A few plasma cells may develop in the cortex and medulla with age, but generally the number of bursal plasma cells remains very low, suggesting some kind of inhibition of B cell terminal maturation. However, in birds surviving IBDV infection the bursa has a remarkably large number of plasma cells before fibrosis is completed.

One perennial question remains: Are there any differences between cortical and medullary B cells? Trafficking of bursal cells between the cortex and medulla is known to occur and there are some differences in phenotype but the functional differences are not fully understood (see Chapter 4 for further discussion). Medullary B cells express surface IgM and a novel primordial germ cell antigen, but the MHC class II antigen appears only on cortical B cells. This makes it difficult to determine the B cell maturation process. During acute involution of the bursa – caused by either IBDV or treatment with testosterone or emetine – T lymphocytes accumulate in the follicles. The more severely damaged follicle has more T cells, suggesting a delicate balance between T and B cells. If B cells are destroyed or emigrate from the follicle, T cells can temporarily replace them. The accumulation of T cells in bursal follicles seems to depend on B cell depletion and not just infection.

Bursal Cortex

The chalice-shaped cortex begins to develop around hatching. Separated from the medulla by the BL of the cortico-medullary border, its surface is covered by fine collagen-rich capsules. The cortex develops exclusively from the mesoderm, unlike the medulla. Mesenchymal reticular cells (MRC) form the supporting cells of the cortex and express vimentin and desmin-intermediate filaments. The three-dimensional meshwork of the MRC is filled with lymphocytes and few histologically defined macrophages. BSDC are lacking in the cortex. In the medulla the ERC form a cellular network and there is no immunocytochemically identifiable extracellular matrix (ECM); unlike the cortex, where MRC produces ECM. The ECM is rich in collagen III and fibronectin, indicating intensive cell migration through the cortex. It is important to note, that the complete absence of ECM in the medulla means cell migration is questionable. Muscle contraction of the bursa possibly contributes to the cell relocation within the medulla and the emptying of lymphatics. The cortex has a rich capillary network adjacent to the cortico-medullary BL and cortex is drained by post-capillaries, which conjoin to form veins in the connective tissue of the fold where the lymphatics emerge (Fig. 2.5(f)).

Peripheral Lymphoid Tissue of the Bursa of Fabricius

Close to the bursal duct one of the ventral folds produces peripheral (secondary) lymphoid tissue (Naukkarinen and Sorvari, 1982) (Fig. 2.5(g)). This tissue is not likely to be a simple continuation of the cloacal lymphoid infiltration, because the fold is flat, wider than others and its colonization by lymphoid precursors is delayed about 1 day. The peripheral lymphoid tissue consists of GC and an interfollicular-dense lymphatic substance that represent B- and T-dependent regions, respectively. This fold may provide communication between the cloacal contents and the bursa of Fabricius (Schaffner *et al*., 1974).

SPLEEN

Origin and Anatomy

The splenic primordium first appears as a mass of mesenchymal cells in the 48-h embryo (Romanoff, 1960). Sinusoids with erythrocytes appear in the mesenchyme at EID 5, granulopoiesis begins from EID 7 with erythropoiesis following at EID 11. In contrast to mammals, the avian spleen is not considered a reservoir of erythrocytes for rapid release into the circulation (Sturkie, 1943). Although not a primary site for lymphocyte antigen-independent differentiation and proliferation, the spleen has an important role in embryonic lymphopoiesis, for it is here that B cell progenitors undergo rearrangement of their Ig genes before colonizing the bursa of Fabricius (Masteller and Thompson, 1994; Chapter 4). At the time of hatching the spleen becomes a secondary lymphoid organ providing an indispensable microenvironment for interaction between lymphoid and non-lymphoid cells. The contribution of the avian spleen to the immune system as a whole may be more important than in mammals because of the poorly developed avian lymphatic vessels and nodes.

The chicken spleen is a round or oval structure lying dorsal, to and on the left side of, the proventriculus (Plate 1.1). One or more small accessory spleens sometimes occur nearby (Payne, 1971). Major splenic development occurs after hatching, following exposure to antigens (Eerola *et al*., 1987). The main blood supply is provided by the *Arteria lienalis cranialis* and *caudalis* with some small branches of the *A. gastrica* and *hepatica* also entering the spleen. The spleen is surrounded by a thin capsule of collagen and reticular fibres; poorly developed connective tissue trabeculae enter the splenic tissue from the capsula. Branches of the splenic artery travel in these trabeculae then enter the splenic pulp. The central artery divides into smaller central arterioles, which also possess a single muscle layer and from this several penicillar capillaries arise. These capillaries lack muscular layers and have stomata. They are surrounded by Schweigger-Seidel (1863) sheaths or ellipsoids (Plate 2.1). There has been much discussion concerning the junction of the penicillar capillaries with the venous sinuses (Nagy *et al*., 2005). The "closed circulation theory" claims that the penicillar capillary is directly connected with venous sinuses whereas the "open circulation theory" claims that penicillar capillaries can open freely into the pulp cords from where blood enters the sinuses between their endothelial cells. Thus the pulp, or Billroth cords, can be regarded as a portion of the circulation between the arterial and venous system. This kind of circulation requires a unique structure that regulates the blood flow through the spleen. The chicken spleen was considered to have an open circulation system (Fukuta *et al*., 1969). However, perfusion fixation and light and electron microscopic studies (Oláh and Glick, 1982) showed that the capillaries which enter the red pulp are continuous with the sinuses and gradually take on their structure. Therefore, in contrast to the mammalian spleen, which has an open circulation, the spleen of the chicken has a closed circulation system.

General Framework

The basic structure is the same as the mammalian spleen. It consists of red and white pulp, the latter being devoid of erythrocytes and predominantly populated by lymphocytes. Consistent

with its less prominent contribution to the oxygen circulation, avian red pulp occupies less space than its mammalian counterpart – 40–45% in birds compared with 76–79% in humans (John, 1994). Together with the meagre capsule and trabeculae, this helps explain the relatively smaller size. Some divergence in splenic architecture is evident in the chicken, where the distinction between red and white pulp is much less-clearly defined. The differences are discussed below.

Red Pulp

The avian spleen begins to function as a haematopoietic organ shortly after the splenic anlage emerges. In most species this function is transient and restricted to the red pulp. After haematopoiesis has ceased red pulp function changes to filtering out circulating senescent erythrocytes. In order to achieve this, splenic stromal elements develop a unique communication with the circulation. Immunohistochemical investigations show that the splenic extracellular matrix is highly complex, with each compartment having a specific composition that contributes to the adhesion and/or migration of leukocytes and resident cells. Reticular fibres, whose basic constituents are types I and III collagen, form a fine supporting network. Unlike human red pulp, tenascin is not expressed in the avian spleen. Tenascin is a large molecule to which lymphocytes can bind via specific integrin receptors; it contributes to lymphocyte migration and exerts immunomodulatory effects (Gumati *et al*., 2003).

Since the chicken spleen has a closed circulation system, capillaries enter the red pulp and join to sinuses. These are lined with a flat endothelium and connected with one another. Sinuses are drained by collecting veins, which fuse to become large trabecular veins that leave the spleen.

Both lymphoid and non-lymphoid cells are recognized in the red pulp. Single T cells are abundant in the sinuses and are $CD8^+$ and TCR$\gamma\delta$ (recognized by mAb $TCR1^+$), although some $CD4^+$ TCR$\alpha\beta1$ or TCR$\alpha\beta2$ cells are also present (Fig. 2.6(a)–(e)). In adult birds most-TCR$\gamma\delta^+$ cells have a heterodimeric CD8$\alpha\beta$ receptor but in embryonic and neonatal spleens, a large proportion of $CD8^+$ TCR$\gamma\delta^+$ cells express only a homodimer of the CD8α chain (Tregaskes *et al*., 1995). From the embryonic spleen a natural killer (NK) cell population of CD8$\alpha\alpha$ cells co-expressing CD25 and 28-4 can be isolated (Göbel *et al*., 2001). Plasma cells expressing each of the Ig isotypes are also present in the red pulp, especially near the large blood vessels. They show strong intracytoplasmic Ig staining and are $CD57^+$ but $chB6^-$ (Mast and Goddeeris, 1998a). In the red pulp many macrophages are present. These are strongly acid-phosphatase positive and stain with mAb -68.1, and -74.2 (Jeurissen *et al*., 1994), KUL-01 (Mast *et al*., 1998) and EIV-E12 (Pharr *et al*., 1995). Expression of MHC class II antigen has been demonstrated on $KUL\text{-}01^+$ cells suggesting a role in antigen presentation. Moreover, these macrophages are capable of phagocytosis since colloidal carbon (Indian ink) is detected in the cells within 24 h of being injected intravenously. Other non-lymphoid cells, such as heterophils, are also scattered throughout the red pulp sinuses.

White Pulp

The spleen is the largest lymphoid organ lacking afferent and efferent lymphatics, like "true" lymph nodes (see later), and can only obtain antigens from the blood circulation. White pulp follows and surrounds the splenic vascular tree (Oláh and Glick, 1982). Chicken white pulp contains morphologically distinct areas: (1) PALS that surround the central arteries, which have visible muscular layers and (2) PWP that surrounds the penicillary capillaries. Schweigger-Seidel sheaths or ellipsoids are embedded in the PWP (Plate 2.1). The PWP is sometimes described as the peri-ellipsoid lymphocyte sheath, analogous to the mammalian marginal zone. GC surrounded by a capsule of connective tissue develop at the bifurcation of the arteries, at the origin of the PALS.

Peri-arteriolar Lymphoid Sheath

Arterioles are surrounded by dense sheaths of T lymphocytes, the PALS. These T cells are $CD3^+$ and most express CD4 and $\alpha\beta_1$TCR molecules. The remaining T cells express CD4 and

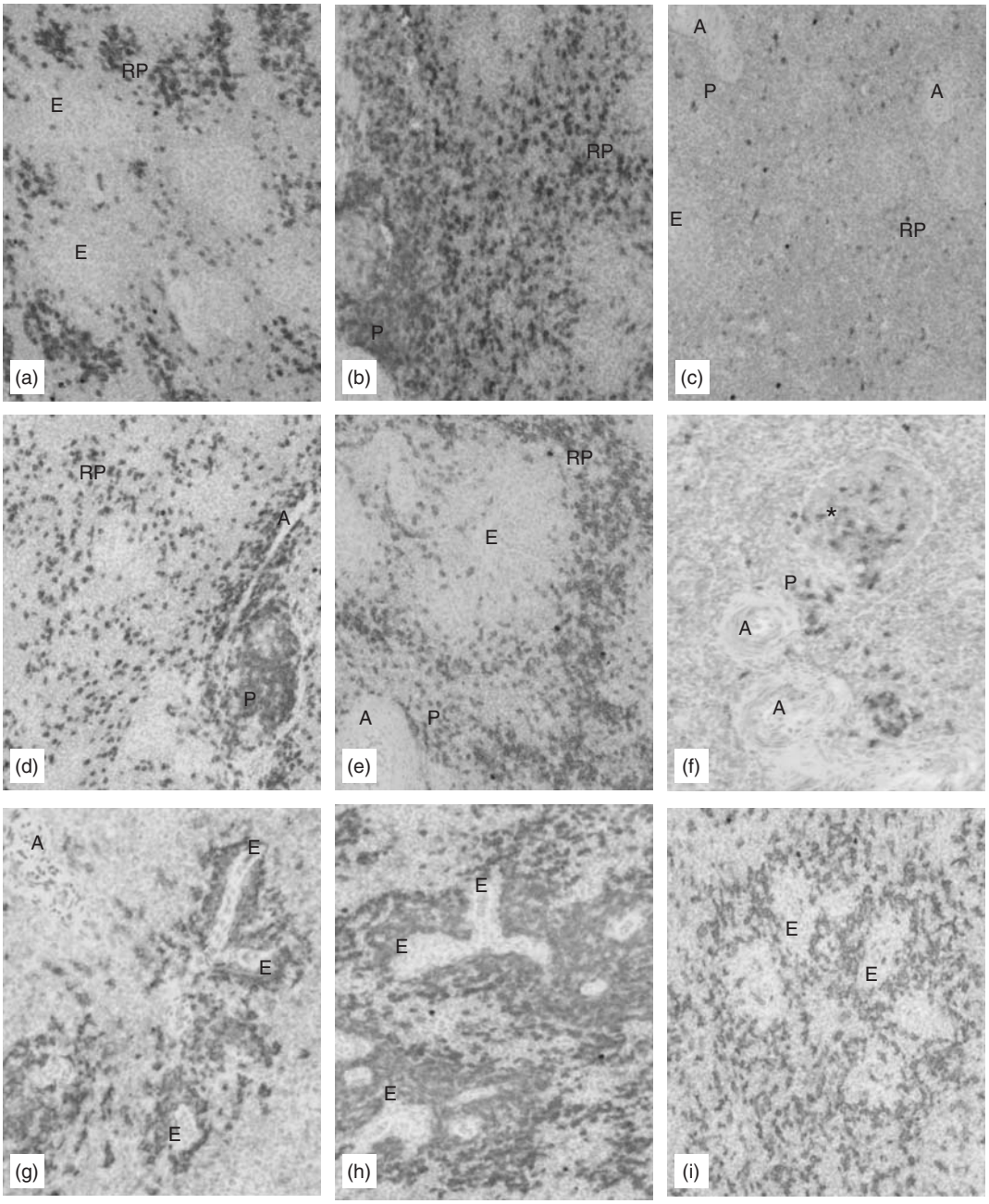

FIGURE 2.6 Detailed structure of the chicken spleen. In the red pulp (RP) both lymphoid and non-lymphoid cells are found. T cells are abundant and express mostly the TCR$\gamma\delta$ (a) or TCR$\alpha\beta$1 (b) and only few TCR$^+\alpha\beta$2 cells (c) are found. Arteries and arterioles (A) are surrounded by peri-arteriolar lymphoid sheaths (P) consisting of T cells expressing CD4 (d), CD8α (e) and germinal centres (asterisk) with CVI-ChNL-74.3$^+$ follicular dendritic cells (f). On sections (g)–(i), ellipsoids and peri-ellipsoid white pulp (E) with CVI-ChNL-68.2$^+$ ellipsoid-associated reticular cells (g) are surrounded by ChB6$^+$ B Cells (h). Outwith the PWP another sheath is formed by CVI-ChNL-74.2$^+$ macrophages (i).

TCR$\alpha\beta_2$ or CD8 and TCR$\alpha\beta_1$ (Fig. 2.6(a)–(e)). In thymectomized chicks depleted of TCR$\alpha\beta_1$ cells, the number TCR$^+\alpha\beta_2$ cells in the PALS increases and partially replaces their functions (Cihak *et al.*, 1991). As in mammals, the CD4$^+$ helper cells play a central role in immune responses and their activation is a prerequisite for the responses of cytotoxic T lymphocytes

(CTL) and B cells. Their central localization around, and in, GC indicates their role in B cell maturation.

In the PALS, interdigitating dendritic cells (IDC) are dispersed between the T cells and are KUL-01$^+$, MHC II$^+$ but CD57$^-$ (Mast and Goddeeris, 1998a). In the PALS the IDC can express S-100 and -74.3 antigens (Fig. 2.6(f)). These cells are likely to be the precursors of FDC, since they are found either scattered or in aggregates, the approximate size of GC (Oláh and Glick, 1982; Gallego *et al*., 1993; Jeurissen *et al*., 1994).

Various compartments of the chicken spleen are involved in humoral responses. The chB6$^+$ cells in the PALS (Fig. 2.6(h)) are CD57$^-$ and most cells express IgM or IgA but only few express IgY. The antigen-specific antibody-producing cells are first located in the PALS, but later most are found in the red pulp (Jeurissen, 1993; Vervelde *et al*., 1993b; Mast and Goddeeris, 1998b).

Ellipsoids and PWP

Penicillar capillaries lack muscular layers, have stomata and are lined with cuboidal endothelium. The mid-portion of the capillary is surrounded by the ellipsoid which is an efficient filtration apparatus with mechanical filtration carried out by the extracellular matrix and phagocytic filtration by cellular components. Cells of the ellipsoid are surrounded by the discontinuous capsule of Schweigger-Seidel sheath (CSS; see Plate 2.1). The basal membrane and the CSS are interconnected with fine reticular fibres, forming a three-dimensional network. Intravenously injected extracellular tracers or antigens leave the circulation through the capillary stomata entering the ellipsoid, whose cells are the first to be exposed to antigens. The CSS provide a mechanical filtering system, preventing tracers and antigens from spreading freely into the PWP.

Penicillar capillaries are surrounded by the cells of the ellipsoid which are reticular cells, round or ovoid in shape (Oláh and Glick, 1982). These cells are also referred to as ellipsoid-associated reticular cells (EARC; Mast and Goddeeris, 1998a), ellipsoidal reticular cells (Kasai *et al*., 1995) or ellipsoid-supporting cells (Nagy *et al*., 2005). The ellipsoidal cells express the antigen CSA-1 and contain heat shock protein (HSP)47 in their cytoplasm, similar to the endothelial cells of the penicillar capillaries (Kasai *et al*., 1995). HSP47 binds to procollagen and may play a role as chaperone, especially for types I, II and IV collagen. Nagy *et al*. (2005) have confirmed that collagen types I and III are produced in the ellipsoids. Using specific markers the ellipsoidal cells have been identified as CD57 $^+$, KUL-01$^-$ (Mast and Goddeeris, 1998a).

Ellipsoid-associated cells (EAC) are highly phagocytic and found at the surface of the ellipsoid (Oláh and Glick, 1982); they express the markers recognized by mAb: -68.2 (Jeurissen, 1991), S-100 (Gallego *et al*., 1993) and E5G12 (Igyarto *et al*., 2007), they are low acid phosphatase positive, and are responsible for the clearance of antigens from the circulation (Fig. 2.6(g)).

The ring of EAC is surrounded by B lymphocytes within the PWP and these cells express chB6 and CD57 (Mast and Goddeeris, 1998a; Fig. 2.6(h)). Around the PWP another ring is formed by acid phosphatase-containing macrophages, which can be identified using mAb-74.2 and KUL-01 (Jeurissen *et al*., 1992; Mast *et al*., 1998; Fig. 2.6(i)).

The Marginal Zone-Equivalent and Antigen Handling

In the mammalian spleen the marginal zone plays a crucial role in the initiation of humoral immune responses. After intravenous injection, antigens are discharged from arterioles into the marginal sinus and localize within the marginal zone. Most antigens are redistributed to the red pulp follicles or PALS. Soluble T-cell-independent type-2 antigens, such as Ficoll are selectively absorbed by marginal zone macrophages. In mice, marginal zone macrophages express pattern-recognition receptors (such as Toll-like receptors, TLR), C-type lectin SIGNR1 (mouse-equivalent of DC-SIGN) and the type-I scavenger receptor MARCO (found in the chicken genome). These receptors show a markedly complementary recognition of pathogens (Mebius and Kraal, 2005). It has been suggested that these macrophages play a critical role in processing particulate antigens into smaller fragments. T-dependent antigens can be redistributed from the marginal zone to the

PALS by marginal zone B cells that, in reacting to antigens, migrate to T cell areas. MHC-restricted antigen presentation to T cells occurs predominantly in the PALS. Each compartment of the spleen has its own population of non-lymphoid cells: macrophages in the red pulp, IDC in the PALS, marginal zone macrophages and FDC in the GC. Being involved in breakdown of antigens, these non-lymphoid cells, can be recognized *in situ* by their enzyme contents. Almost all non-lymphoid cells contain acid phosphatase, while non-specific esterase is only found in marginal zone macrophages.

In contrast, in the chicken spleen, a corona and marginal zone cannot easily be distinguished morphologically. A compartment that is functionally similar, with respect to antigen handling and reaction to mitogenic antigens, has been described by Jeurissen *et al*. (1992). This compartment is made up of the complex ellipsoidal sheath of reticular cells, a sheath of B cells with a ring of macrophages around it. EAC are responsible for clearance of antigens from the circulation and consequently these become localized in the ellipsoids. This process is independent of the nature of the antigens, i.e. they can be: live or dead; soluble or particulate; thymus-dependent or -independent type-1 and -2; with or without an Fc tail. In this respect, the ellipsoid resembles the marginal sinus of the mammalian spleen.

The nature of the antigen influences subsequent transport from the ellipsoids to the B cells and surrounding ring of macrophages. Colloidal carbon has frequently been used to investigate the fate of antigen but it does not evoke immune responses and so it is not clear that the observations reflect handling of real antigens. Carbon particles initially localize in the ellipsoid, and the majority are redistributed to the red pulp macrophages. Small amounts are found in the PALS around, but not inside, GC. During the 1960s and early 1970s White and colleagues (White *et al*., 1969, 1975) carried out functional studies which clearly showed the transport of fluorescently labelled antigens or immune complexes from PWP towards the PALS and GC. They named these antigen-carrying cells "dendritic-like cells": these are probably identical with the EAC later described by Oláh and Glick (1982). Light and electron microscopical studies, using different intravenously injected tracers, suggested that an EAC, after trapping antigen, detaches from the ellipsoid and appears in the PALS around the GC, then subsequently in the GC (Oláh and Glick, 1982, 1984; del Cacho *et al*., 1995; Gallego *et al*., 1997). This migration pathway led to the hypothesis that the EAC could be the precursor cells of the IDC and FDC (Oláh and Glick, 1982). Recently the relationship between the EAC and FDC has been studied by intravenously injecting bacterial β-galactosidase, which was taken up exclusively by the EAC and later appeared in T and B cell aggregates and FDC of the existing GC. A recently produced mAb, E5G12, recognizes both EAC and FDC and double staining for the E5G12 antigen and β-galactosidase demonstrated that the EAC carries β-galactosidase to the PALS and GC. Therefore, EAC could be precursors of the FDC in splenic GC; but not of the FDC in GC that located elsewhere (Igyarto *et al*., 2007). The question remains whether the population of -68.2$^+$ and E5G12$^+$ cells may in fact be one or more subpopulations each with its own antigen specificity. When multiple antigens were intravenously injected differences in localization were found (Jeurissen, 1991). Two hours after simultaneous injection of FITC-Ficoll and peroxidase anti-peroxidase (PAP) immune complexes, both antigens colocalized in -68.2$^+$ cells. In contrast to PAP, FITC-Ficoll continued to be detected on -68.2$^+$ cells in the ellipsoid at later timepoints, whereas PAP migrated via the PALS to the GC on cells lacking the -68.2 marker. Again this suggests that either multiple subpopulations of EAC exist and, depending on the antigen, these cells either migrate into the PALS or not, or that antigens are given to other cells. Cells may lose their markers in the process of migration and differentiation, which makes tracking of them complex. For all these, mAbs specific for chicken macrophages, dendritic and reticular cells the molecules that are being recognized remain undefined and this does not facilitate progress in this field. Although it is generally accepted that T-dependent antigens like KLH and T-independent type-1 antigens like lipopolysaccharide (LPS) are mainly absorbed by red pulp macrophages. T-independent type-2 antigens like Ficoll do not relocalize after entry into the ellipsoid, but remain in the ellipsoid-associated cells for longer periods, and immune complexes are first found in the ellipsoid and relocalize to the PALS, more specifically on precursor and mature FDC, and they remain present in mature GC. The localization of preformed immune

complexes is dependent on the Fc tail as pepsin diminishes localization. Immune complexes are also first found in the ellipsoid and relocalize to the PALS, more specifically on precursor and mature FDC, and they remain present in mature GC.

Non-lymphoid cells which handle antigen can be influenced by viruses, bacteria and bacter-ial products. After administration of live viruses, such as human adenovirus or Marek's disease virus, the sheath of ellipsoidal cells becomes disrupted and a part of the EAC population migrate to the PALS (Jeurissen *et al*., 1989b; Oláh *et al*., 1990). High doses of LPS or bacteria with LPS have strong effects on the -74.2^{+} macrophages around the PWP. After intravenous injection of LPS macrophages around the PWP disappear, as do the B cells in the PWP, but then these cells reappear after 48 h. A similar relocalization of marginal zone B cells to B cell follicles has been reported for mice stimulated with LPS; it was shown that LPS downregulated expression of retention signals ($S1P_1$ and $S1P_3$) on marginal-zone B cells (reviewed by Mebius and Kraal, 2005).

The complex of ellipsoids, B cell sheath and its surrounding macrophages can be considered as functional homologues of the marginal zone. This may not be general since the formation of ellipsoids and/or marginal sinuses varies between species. Well-organized ellipsoids, such as those in chickens, have also reported been for dog and cat but not for rodents, which have marginal zones instead. In contrast to the mouse, the human spleen has both ellipsoids (poorly developed) and an inner and outer marginal zone (Jeurissen, 1993; Kasai, *et al*., 1995; Mebius and Kraal, 2005).

In the spleen, both innate and adaptive immune responses can be efficiently mounted, making it an important organ for immune regulation. Based on the localization of various lymphocyte and non-lymphoid cells in the spleen, the PALS seems most involved in the adaptive immunity, whereas the ellipsoids and PWP are involved in both innate and adaptive immune responses.

GUT-ASSOCIATED LYMPHOID TISSUES

Chickens lack encapsulated lymph nodes like those of mammals and instead develop diffuse lymphoid tissue, wherever they are antigenically stimulated in the body. Since mucosae are primary targets for antigens, chickens have extensive mucosa-associated lymphoid tissues. These include the GALT dealt with here and in Chapter 13, the respiratory-associated lymphoid tissue, described in Chapter 14, and the reproductive-associated lymphoid tissue, described in Chapter 15.

Along the entire intestinal tract small lymphoid accumulations, solitary nodules or follicles occur in addition to aggregated lymphoid nodules. Scattered along the tract are several defined lymphoid tissues, CT (Plates 1.1 and 2.2(a)), PP (Plate 2.2(a)) and the bursa of Fabricius (as described above). Defined lymphoid tissues that have been less-well studied include Meckel's diverticulum (Plate 2.2(b)), oesophageal and pyloric tonsils and lymphoid accumulations in the roof of the pharynx and in the cloaca.

During the embryonic and postnatal phases, most organized lymphoid tissues begin to develop independently of antigen stimulation, since they form at predetermined sites before hatching. However, further maturation of the lymphoid tissues is antigen driven, as determined in studies using germ-free chickens and Japanese quail (Hedge *et al*., 1982). Compared to conventional birds, there were no consistent differences in weights of the bursa, thymus or spleen, but markedly reduced lymphoid tissues in the GALT and a complete absence of lymphoid follicles in the CT of germ-free birds (Hedge *et al*., 1982).

The development of GALT starts in the lamina propria of the villus. Each villus consists of a core of connective tissue fibres, smooth muscle fibres, nerves, and blood and lymph vessels. Around this core lymphocytes intermingle with macrophages in the lamina propria, and the more lymphocytes that arise in the villus the more they form distinct areas of B and T cells. Generally T cell areas are located towards the villus core (Jeurissen *et al*., 1994). The next stage

in development is the formation of B cell follicles and GC, which appear within the T cell areas of the deep and mid-portion of the lamina propria (Hoshi and Mori, 1973). The lamina propria is bordered at the luminal side by a thick and continuous basement membrane, and situated on this membrane is a layer of columnar epithelial cells. Mucus-producing goblet cells are interspersed between these epithelial cells.

The stroma of the villus consists of a cell-rich extracellular matrix, smooth muscles and columnar epithelial cells which express a variety of cell determinants, e.g. region-specific carbohydrates or lectins (Alroy *et al*., 1989; Vervelde *et al*., 1993a) and antigens similar to MHC class IV (BG) antigens (Miller *et al*., 1990). Mucosal epithelial cells synthesize the polymeric Ig receptor (pIgR) to which polymeric IgA binds. Receptor:Ig complexes are endocytosed and subsequently transcytosed to the apical surface where secretory IgA is released into the mucosal lumen by proteolytic cleavage of the receptor ectodomain. A gene encoding chicken pIgR has been identified and shown to be expressed in the jejunum (Wieland *et al*., 2004). TLR that detect invading pathogens and mount an anti-microbial response are expressed along the chicken intestinal tract, especially TLR1/6/10 and TLR3, and the mRNA expression patterns do not differ along the various parts of the intestine (Iqbal *et al*., 2004; see also Chapter 13). Expression of TLR on the gut epithelium remains to be elucidated.

The lymphoid tissues have a well-organized structure with an interfollicular space where T cells accumulate, while B cells form follicles and GC; this is all overlaid by a specialized FAE. Apart from organized lymphoid tissues, single lymphocytes and aggregates in the lamina propria and epithelium are found along the intestinal tract. The lamina propria contains both B and T cells. The majority of T cells express CD4 and TCR$\alpha\beta_1$. Thymectomy and depletion of TCR$\alpha\beta_1^+$ cells severely compromises IgA production in the gut, serum, bile and lung lavage fluids (Cihak *et al*., 1991). CD8$^+$ TCR$\gamma\delta^+$ cells and CD4$^+$ TCR$\alpha\beta_2^+$ cells are scattered throughout the lamina propria (Bucy *et al*., 1988; Cihak *et al*., 1991; see Chapter 13). TCR$\alpha\beta_2^+$ cells are rarely evident in the small intestine but their relative frequency is higher in the CT. IgM$^+$, IgA$^+$ and IgY$^+$ plasma cells are found in the villi with a predominance of IgA-producing cells in the lamina propria. IgM$^+$ and IgY$^+$ plasma cells are mostly located between the crypts, whereas IgA$^+$ plasma cells are often scattered from the crypts to the villi tips. Macrophages are located in the sub-epithelial space; they express MHC class II antigen and contain acid phosphatase. Although age does not influence the characteristic distribution pattern of these lymphocytes, the number of cells increases in older birds (Jeurissen *et al*., 1989a).

A heterogenous population of intra-epithelial leukocytes (IEL) resides among the epithelial cells. Studies on chickens dosed with ^{3}H-thymidine demonstrated that IEL represent lymphocytes that migrate from the lamina propria into the epithelium (Bäck, 1972b). IEL are found in the stratified epithelium of the oesophagus and in the columnar epithelium lining the gut, but rarely in the epithelium of the proventriculus. Few IEL are detected at hatching and their numbers increase greatly with age; however, in old birds the number decreases significantly, returning to the level in young chicks (Vervelde and Jeurissen, 1993). IEL consist of a heterogeneous population of cells that are located in the basal and apical part of the epithelium (Bäck, 1972a; Vervelde and Jeurissen, 1993). CD3$^+$ IEL are predominantly CD8$^+$ cells and most express TCR$\gamma\delta$ (Bucy *et al*., 1988). However, about 30% of the CD8$^+$ IEL lack CD3 and co-express the 28-4 antigen (Göbel *et al*., 2001). These cells show NK-cell like activity. The chB6$^+$ cells in the epithelium are recognized by the mAb HIS-C1, specific for chB6, and either L22 or 11G2, specific for chB6a and chB6b, respectively (Vervelde and Jeurissen, 1993; Pink and Rijnbeek, 1983; Veromaa *et al*., 1988), and do not express Ig. Their origin remains unclear, since bursectomy does not affect the IEL population (Bäck, 1970). Another, or maybe the same, ill-defined cell within the epithelium is the globular leukocyte. This cell is slightly larger than the IEL and contains granules, like the chB6$^+$ cells, with a different absorbance spectral curve than mast cells, and are morphologically similar to NK cells (Kitagawa *et al*., 1988). During the first week after hatching, intestinal lymphocytes already segregate not only anatomically but also functionally into lamina propria B cells, T-helper cells and epithelial cytotoxic/suppressor cells.

Follicle-Associated Epithelium or Lymphoepithelium

Epithelium covering organized lymphoid tissue, such as PP, CT, bursa of Fabricius and Meckel's diverticulum, is not arranged as in the villi and intestinal glands. This epithelium is characterized by irregular microvilli, numerous apical tubules, vesicles and vacuoles and dense cytoplasm. Moreover, this epithelium is often flattened, lacking mucus-producing goblet cells. The cells contain alkaline phosphatase, whereas the villous columnar epithelium does not. Lymphocytes penetrate the epithelium to form a lymphoepithelium (Fig. 2.1(c)), except in the bursa of Fabricius. Breaks in the epithelium allow extrusion of lymphocytes into the lumen. One characteristic of the specialized epithelium is that it contains cells that are highly absorptive M cells, and these cells show very efficient pinocytotic activity (Bockman and Cooper, 1973; Burns, 1982). Chicken M cells can be visualized in Meckel's diverticulum and CT using lectins specific for *N*-acetyl-D-galactosamine (SBA), α- L-fucose (AAA) and *N*-acetyl-D-glucosamine (WGA). Only a few studies have been carried out on the actual uptake of antigen and these were limited to the uptake of Indian ink and ferritin (Bockman and Cooper, 1973; Burns, 1982; Jeurissen *et al*., 1999). Although uptake of ferritin by the M cells was observed, uptake by other epithelial cells also occurred; it was concluded that the phenotypes and functions of chicken M cells and regular epithelial cells are less clear cut than their mammalian equivalents (Jeurissen *et al*., 1999).

Peyer's Patches

Lymphoid aggregates with characteristics of mammalian PP have been described (Befus *et al*., 1980; Burns, 1982). Up to six PP can be detected widely scattered in the intestine, with the exception of one that is consistently found anterior to the ileocaecal junction (Befus *et al*., 1980; see Plate 2.2(a)). Specific features include thickened villi, a follicular structure, a specialized epithelium containing M cells, active antigen uptake development in the embryo and age-associated involution. GC, subepithelial zone and interfollicular areas are similar to those in the CT. The subepithelial zone, where macrophages are more prevalent, is B cell dependent. The interfollicular zone is T cell dependent and almost all T cells express TCR$\alpha\beta_1$ with the majority being $CD4^+$ cells (Bucy *et al*., 1988). Few TCR$\gamma\delta^+$ cells (<5%) are evident in the PP. Mostly IgY^+ and fewer IgA^+ and IgM^+ plasma cells are found in the GC and interfollicular areas. The luminal border of the epithelium is positive for IgA and IgY but not IgM (Burns, 1982).

Muir *et al*. (2000) showed that, besides the bursa, the PP and CT also contain precursor B cells which, upon transfer into bursectomized chickens, repopulate the intestinal lamina propria with IgA^+ plasma cells, signifying a role for the GALT as a primary lymphoid tissue.

Caecal Tonsils

CT are located at the proximal end of each of the caecal pouches (Plates 1.1 and 2.2(a)). The CT primordium appears at about EID 10 and lymphocytes are present by EID 18 (Payne, 1971). During the first week after hatching the number of lymphocytes increases and GC appear in the second week, their number increasing with age. In chickens and quails reared in a germ-free environment no GC were evident in the CT and lymphoid tissue was markedly reduced suggesting that the gut flora is essential to stimulate full development (Hedge *et al*., 1982). A suspension of fine particles applied to the cloacal lips can reach the CT by retroperistalsis, similar to that described for the bursa of Fabricius, suggesting that this could be important for antigen sampling (Sorvari *et al*., 1977).

The general structure of the CT is comparable to the structure of the PP and it includes a specialized lymphoepithelium, a subepithelial zone, GC, and interfollicular areas. The subepithelial zone has a mixed appearance with mostly $chB6^+$ (Fig. 2.7(a)), IgM^+ B cells, some IgY^+ B cells and occasionally IgM^+ and IgA^+ plasma cells, and few $CD4^+$ cells and $CD8^+$ T cells expressing the γδ- or αβ-TCR (Fig. 2.7(b), (c), (e) and (f)). Mononuclear phagocytes can be found throughout the tonsils, but are most prevalent directly under the epithelium (Fig. 2.7(d)).

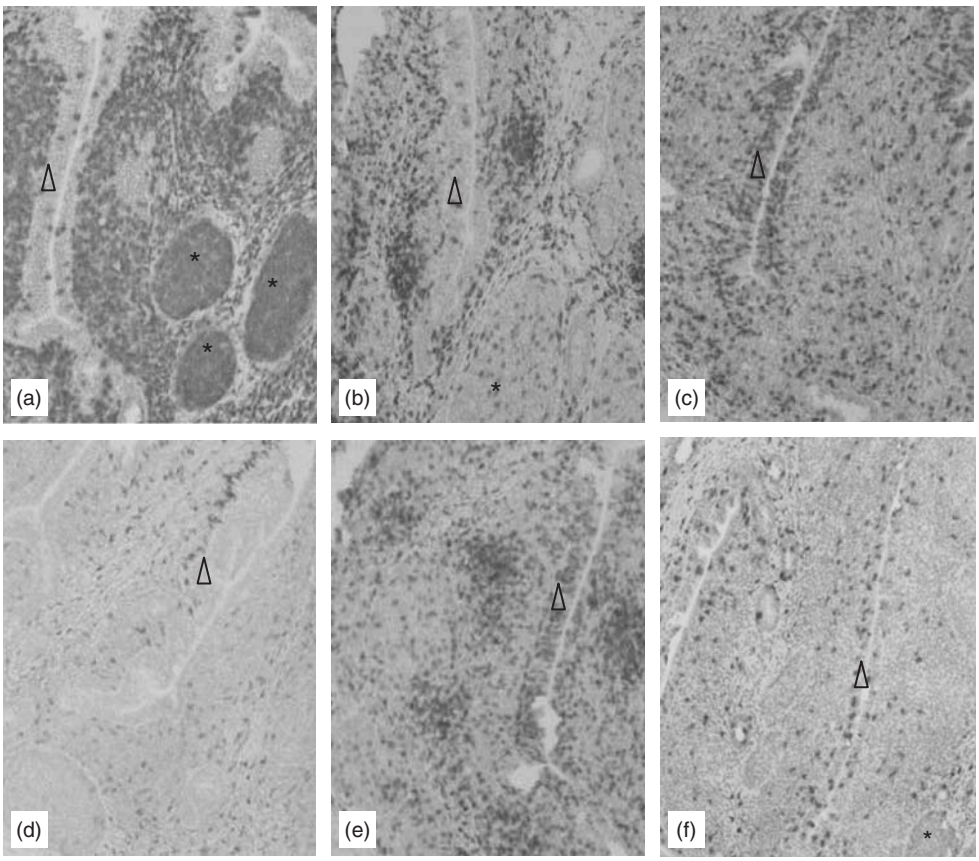

FIGURE 2.7 Detailed structure of the chicken caecal tonsil. Caecal tonsil; luminal side is located at the top. ChB6$^+$ B cells (a) occur in the lamina propria, germinal centres (asterisk), and few in the epithelium (open triangle). CD4$^+$ cells (b) are located in the centre of the villi, in the interfollicular areas, and few in germinal centres. C8α^+ cells (c) are most prevalent in the epithelium and scattered through the lamina propria. KUL-01$^+$ macrophages (d) occur scattered through the lamina propria and form a lining under the epithelium. The location of $\alpha\beta$1-TCR$^+$ cells (e) is comparable to both CD4$^+$ and CD8$^+$ cells, whereas $\gamma\delta$-TCR$^+$ cells (f) are located mostly in the epithelium and subepithelial zone.

The interfollicular T cell dependent area consists mainly of CD4$^+$ TCR$\alpha\beta_1{}^+$ cells (Bucy *et al.*, 1988; Jeurissen *et al.*, 1994). More detailed information on the CT can be found in Chapter 13.

Meckel's Diverticulum

The remnant of the yolk stalk is an appendage of the small intestine, halfway along the jejunum and called Meckel's diverticulum (Plate 2.2(b)). After hatching significant amounts of yolk pass into the intestine providing a food supply for the chick. The remnant of the omphalomesenteric, or vitelline duct, which forms an intermediate between the yolk sac and the gut of the embryo, persists for about 5 weeks after hatching (Latimer, 1924).

The proximal end of Meckel's diverticulum opens as a slit into the small intestine on a flat, elongated papilla. Two longitudinal folds formed by submucosal connective tissue line the opening. These folds are covered by intestinal epithelium. The wall of Meckel's diverticulum consists of four histologically distinct layers. A serosa covers a thick layer of connective tissue which may correspond to the subserosa of the intestine. Internal to this layer bundles of smooth muscles are arranged around Meckel's diverticulum. On the luminal side of the muscle

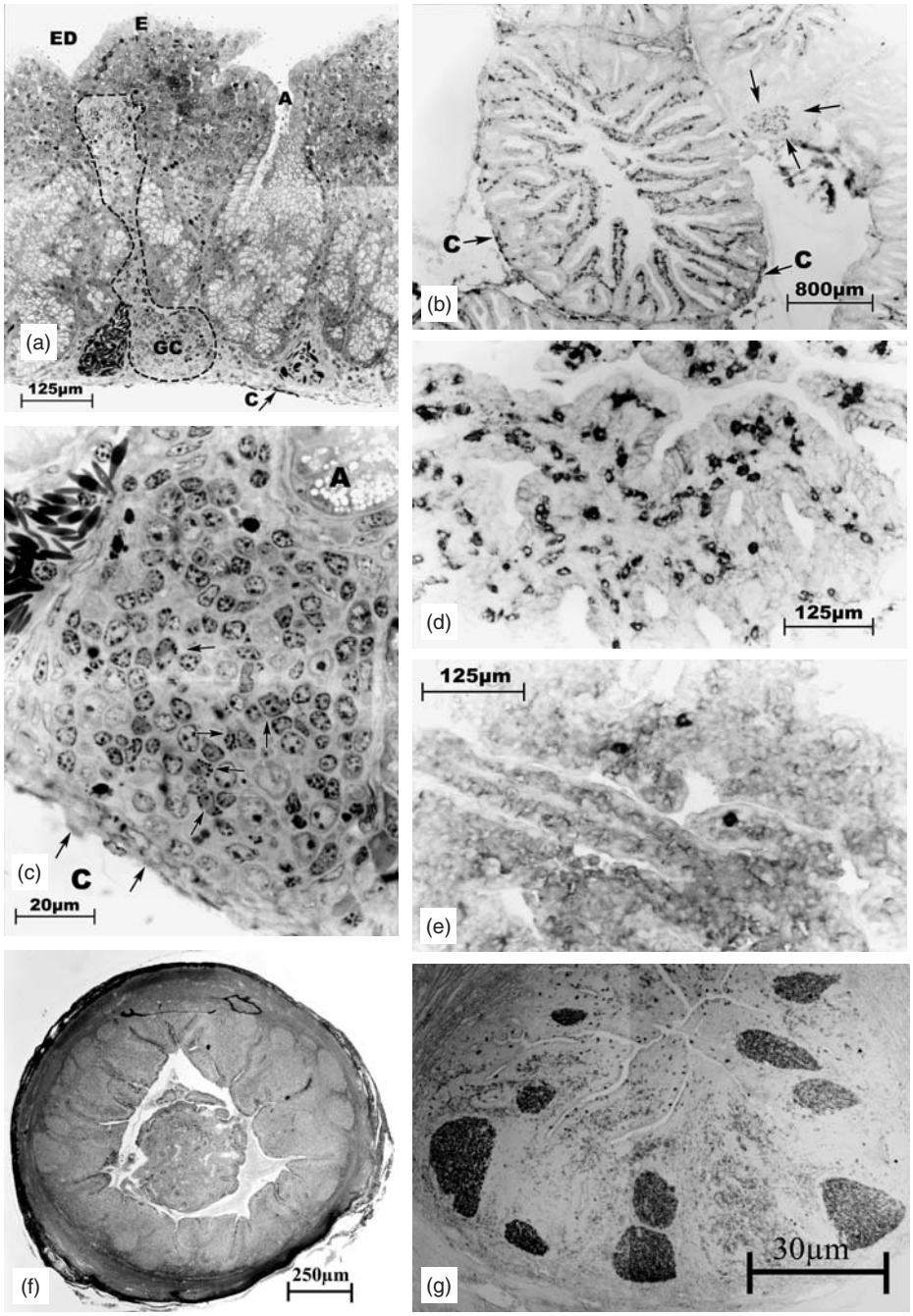

FIGURE 2.8 Detailed structure of the chicken Harderian gland. (a) The lymphoid region of the Harderian gland is divided into two parts: one is under the epithelium (E) of the main excretory duct (ED) which almost exclusively contains plasmablasts and plasma cells. The other is dense lymphoid tissue (outlined) which includes GC. C: capsule; A: secretory acinus. (b) Anti-IgM recognizes plasma cells in the wall of the secretory duct. No plasma cells are close to the capsule and the secretory acinus. An arrow indicates one germinal centre in which the follicular dendritic cells are surface IgM positive. (c) Detail of Fig. 2.8(a). In the dense lymphoid tissue a germinal centre is formed close to the C. In the centre of the GC several FDC are shown (arrow). (d) Immunostaining shows that under the secretory duct epithelium a large number of IgA-producing cells occur. (e) Scattered IgY positive cells occur in the Harderian gland. (f) Meckel's diverticulum from a 5-week-old chicken. The GC occur around the whole diverticulum, unlike in the PP and CT where they are located distal to the mesenteric side of the intestine. (g) Details of Meckel's diverticulum. Staining for chB6 reveals that the majority of B cells are in the germinal centres but scattered B cells occur in the surface epithelium.

the connective tissue contains large vessels and ganglions. This layer folds into the lumen of Meckel's diverticulum and its surface is covered by columnar epithelium with mucus-producing goblet cells. At hatching, no lymphoid tissue is found in the Meckel's diverticulum; however, during regression of the yolk sac in the first 2 weeks after hatching, myelopoietic tissue appears distal to the end of the diverticulum, whereas lymphopoietic tissue appears more proximal to the intestine, in the subepithelial connective tissue. In the myelopoietic tissue, three zones are recognized on the basis of their cellular content. The zone closest to the lumen of the yolk sac consists of monocytic cells, then a zone with large numbers of undifferentiated blast cells while the largest zone consists almost entirely of immature granulocytic cells. These granulocytic cells produce two types of granules, one is large, electron dense and homogenous, whilst the other is smaller and filled with a finely granulated substance with a visible central crystalline structure. Mature granulocytic cells are rare (Oláh and Glick, 1984).

After hatching, the epithelium growing into the subepithelial connective tissue forms longitudinal folds and small groups of lymphoid cells accumulate beneath the epithelium. Single $CD45^+$ cells infiltrate the epithelium and connective tissue. Single B cells that are IgM^+, but not IgY^+ or IgA^+, are located in these infiltrates. Mononuclear phagocytes are found as single cells dispersed over the Meckel's diverticulum. Jeurissen *et al.* (1991) injected colloidal carbon at EID 10 into the yolk and studied absorption during the first 2 weeks after hatching. Carbon particles were first located in the epithelium and subsequently in subepithelial layer and the lamina propria, showing that yolk-derived antigen can be taken up. Brambell (1970), however, found that maternal antibodies in the yolk are transmitted to chicken embryos via the veins in the yolk sac wall and not via the intestine, indicating that a dichotomy between absorption of serum proteins and foreign antigens might exist.

The lymphoid tissue in Meckel's diverticulum gradually increases with age and fills the folds. Concomitantly, the number of goblet cells is reduced, the epithelium is infiltrated by lymphocytes, and inside the folds clusters of lymphoblasts are formed around DC. After 2–3 months GC appear and large numbers of GC are located close to the muscle layer (Oláh *et al.*, 1984; Jeurissen *et al.*, 1988b; Fig. 2.8(f) and (g)). Dispersed throughout the Meckel's diverticulum are IgA and IgY plasma cells and $sIgA^+$ and $sIgY^+$ cells. At a later age, both B and T cell areas can be distinguished. The T cell areas are situated adjacent to GC whereas B cell areas are generally beneath the epithelium (Jeurissen *et al.*, 1988b; Fig. 2.8(g)). The anatomical resemblance with CT and PP is clear; however, the development of GC appears to be later which could be due to lower exposure to gut antigens. The large number of plasma cells is reminiscent of the Harderian gland; however, development of the latter precedes that of the Meckel's diverticulum (Glick, 1978; Oláh *et al.*, 1984).

Oesophageal and Pyloric Tonsils

Although the existence of lymphoid tissue at the junction of the oesophagus and proventriculus was described over 30 years ago (Hodges, 1974), a detailed description was provided much later (Oláh *et al.*, 2002a). Like the CT, the oesophageal tonsils are permanent lymphoid structures located at the junction between the oesophagus and proventriculus. The oesophagus forms six to eight longitudinal folds with lymphoid tissue located at the distal ends of these folds, where it forms isolated units or tonsils. The number of units is identical to the number of folds, suggesting they are stable structures. The tonsillar unit consists of a crypt, beginning at the bottom of a fold, and the surrounding lymphoid tissue. These units are restricted to the lamina propria and do not occupy submucosal layers like PP. Lymphoid cells infiltrate the stratified squamous epithelium transforming it into a lymphoepithelium. Strongly positive $chB6^+$ B cells form GC, and many weak $chB6^+$ cells occur in the interfollicular T cell areas, located between and above the GC beneath the epithelium.

The significance of this lymphoid tissue is 2-fold: firstly, it is the only substantial lymphoid accumulation located in the digestive tract cranial to the stomach and is thus exposed to undigested environmental antigens; secondly, it may participate in B cell development like PP.

Oláh *et al.* (2002a) speculated that the tonsils function as a "gate" for environmental antigens, through which antigens or allergens continuously stimulate the immune system. The importance of this organ to the development of oral vaccines needs to be investigated.

Recently we identified a tonsil structure in the pyloric region (Nagy and Oláh, 2007). A unique histological feature of the pyloric tonsil is that it forms a complete ring at the exit of the stomach. This suggests it has an important sentinel role at the entry to the duodenum (Nagy and Oláh, 2007). The large number of GC and the interfollicular regions consist of B and T cells, respectively, indicate that the pyloric tonsil is a well-organized GALT.

HARDERIAN AND CONJUNCTIVAL-ASSOCIATED LYMPHOID TISSUES

The major eye-associated lymphoid tissues are located in the Harderian gland (HG) and conjunctiva of the lower eyelid (see Plate 2.2(c)), although scattered lymphocytes and plasma cells can be found in the lachrymal gland and other connective tissues around the eye. In specified pathogen-free chickens, conjunctiva-associated lymphoid tissue is only found occasionally, but is constitutively present in conventionally reared poultry especially in turkeys (Fix and Arp, 1989; Jeurissen *et al.*, 1989a).

The HG is an ectodermally derived, exocrine tubuloacinar gland located in the orbit behind the eye (Plate 2.2(d), Fig. 2.8(a) and (b)) and is responsible for lubricating and maintaining the nictitating membrane. Structurally an excretory duct links the gland to the nictitating membrane, and the gland itself is divided into a head and body part, based on differences in the surface epithelium and underlying lymphoid organization (Oláh *et al.*, 1996). The excretory duct is invaginated into the head of the gland and shows a classical FAE or lymphoepithelial organization, which continues into the central canal of the gland. At the border of the invaginated excretory duct and the central canal the secretory acini appear and their gradual increase displaces the dense lymphoid tissue. The lymphoid tissue of the HG can be divided in two major histologically different areas: firstly, the FAE with lymphoid tissue and GC in the head of the HG; secondly, the body of the HG, which form the significantly larger part of the gland containing large numbers of plasma cells in different stages of maturation. Plasma cells are closely associated with the main excretory duct and secretory ducts originating from the acini. Plasma cells cross the BL of the ducts and appear between the epithelial cells, changing the cytoskeletal pattern of the epithelium.

The head of the gland shows the structure of a typical secondary lymphoid organ with B cell-dependent GC (Fig. 2.8(c)), FAE and T cell-dependent interfollicular regions with scattered T cells, and macrophages. The body of the gland contains many B lymphocytes and plasma cells and, depending on the level of development, B and T lymphocytes are clustered in separate areas.

Studies by Bang and Bang (1968) demonstrated lymphocytic infiltrates in the HG of germ-free chickens, indicating that lymphoid tissue within the HG might be induced without microbial stimulation, although the presence of inflammatory substances in the air cannot be excluded. Single and small groups of $CD45^+$ leukocytes are found in the connective tissue of the glandular lobes of 5-day-old chicks (Jeurissen *et al.*, 1989a). These leukocytes comprise $chB6^+$ B cells, macrophages, interdigitating cells, and heterophils (Jeurissen *et al.*, 1989a; Bang and Bang, 1968). As chickens age, leukocytic infiltrates gradually increase in size and, depending on the development B and T lymphocytes, are clustered in separated areas. The majority of the T cells are $CD4^+$ and $TCR\alpha\beta_1^+$ with few $CD8^+$ $TCR\gamma\delta^+$ cells scattered around. Leukocytes are mostly absent from the epithelium. The number of plasma cells increases tremendously with age and these cells are found near tubular ducts and inter-alveolar connective tissue. The uniqueness of the HG is the large number of plasma cells which are capable of proliferation *in loco* (Savage *et al.*, 1992; Scott *et al.*, 1993). This phenomenon is age-dependent and rates of high proliferation are evident in 6- to 8-week-old chickens.

Discrepancies exist regarding Ig expression of the B cells. Oláh *et al.* (1996) reported numerous IgM- (Fig. 2.8(b)) and IgA-producing (Fig. 2.8(d)) plasma cells but rarely IgY^+ plasma cells, whereas Jeurissen *et al.* (1989a) described IgY^+ plasma cells (Fig. 2.8(e)), including IgY in the overlying epithelium, but only in birds older than 6 weeks of age. Jalkanen *et al.* (1984) reported more cytoplasmic(c)IgY^+ cells in 10-week-old chickens than $cIgM^+$ and only a small number of $cIgA^+$ cells. In embryonally bursectomized chickens (at 60 h of incubation) the number of $cIgY^+$ and $cIgM^+$ cells was halved, but the most striking effect was the significant loss of $cIgA^+$ cells compared to intact controls. It is not clear if the decrease in $cIgA^+$ cells was compensated by an increase of IgM^+ cells. In bursectomized chickens the number of GC was also significantly reduced. Although Ig-producing cells were still present none of the bursectomized birds showed any evidence of the production of specific antibodies after four immunizations. The variable findings concerning the presence of different Ig isotypes could be related to age, health status and environmental stimulatory agents. Although the HG is used as a source for IgA, IgY^+ plasma cells are found and their activity is reflected in their contributions to tear fluid. The respective concentrations per ml are 0.8 mg IgM, 0.2 mg IgA and 2.3 mg IgY. Almost all Ig in tears is produced locally, because surgical removal of the HG abrogates IgM and IgA levels and reduces IgY levels to 0.5 mg (Baba *et al.*, 1988).

The route of uptake, processing and presentation of environmental antigens which lead to a humoral antibody in tear fluid is still not fully understood. Survashe *et al.* (1979) suggested that the immune response in the HG is initiated in the lymphoid tissue surrounding the opening of the gland duct to the nictitating membrane, whereas others have suggested that, in the turkey at least, uptake of antigen occurs in the lower eyelid and processing in the conjunctival-associated lymphoid tissue subsequently leading to plasma cells in the HG (Fix and Arp, 1989, 1991). More detailed information on antigen uptake, and the effectiveness of antigen administration via various ocular routes, is described in Chapter 14.

MURAL LYMPH NODE

With regard to the lymphoid system, two major phylogenetic events emerged in birds. One was the appearance of a rudimentary lymph node – a major step towards the generalization of the local immune response – and the other was the emergence of the GC, in which the antigen-specific B cells proliferate, Ig isotype switching takes place and memory B cells are formed. These two evolutionary steps make the avian and mammalian lymphoid (immune) systems highly comparable, both structurally and functionally.

The first detailed histological description of the avian lymph node was published by Biggs (1957). He proposed that avian mural lymph nodes (MLN) are normal structures and not ectopic lymphoid accumulations, so they must be considered part of the avian lymphoid system. Ectopic lymphoid accumulations would destroy normal structure unlike in the MLN (Biggs, 1957). Generally, the MLN are associated with the deep lymphatics, which course together with the femoral, popliteal and posterior tibial veins; however, very small lymphoid accumulations also occur along the wing vein. The emergence of the MLN usually takes place after 6 weeks of age and persists in the adult.

There are several anatomical and histological differences between a "true" lymph node (mammal) and the MLN (avian). The former anatomically interrupts the lymph flow, while the MLN is associated with the lateral side of the lymphatic, hence the lymph flow is not interrupted (Fig. 2.9(a)). In the largest MLN, lymph sinuses form a bypass, in which the lymph flow is, possibly, very slow. The basic histological distinction is the absence of reticular fibres and macrophages in lymph sinuses of the MLN. In the "true" lymph node reticular fibres and macrophages contribute to mechanical and biological filtering of afferent lymph, respectively. The appearance of the MLN lacking a filtering system suggests an evolutionary stage in development of the lymphoid system. Nevertheless, a foot-pad injection of antigen or phytohaemagglutinin (PHA) enlarges the MLN located along the posterior tibial vein indicating that antigen

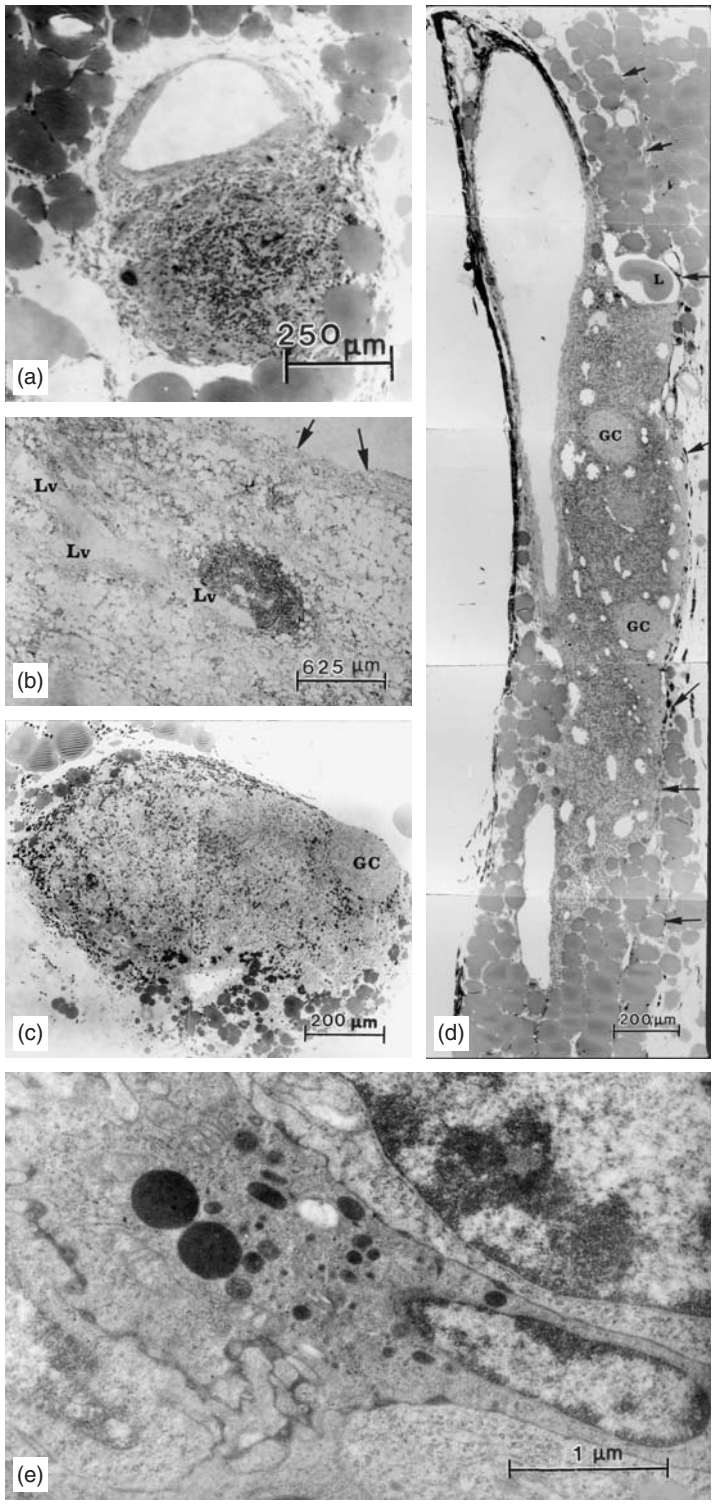

FIGURE 2.9 Details of the structure of the avian MLN. (a) *Type I*: The MLN is associated with the lateral side of the lymphatic. Adipocytes surround the uncapsulated node. (b) The MLN is embedded in adipose tissue. The upper right corner shows the edge of the border of the lipid compartment (arrow), Lv: lymphatic vessel. (c) *Type II*: The MLN with a GC. Many red blood cells are located around the node. (d) *Type III*: Lymphoid tissue fills the space between the lymphatic and the loose connective sheath (arrow), hence the lateral side of the MLN is capsulated while the proximal and distal ends are uncapsulated. GC are embedded in the dense lymphatic tissue. Lymph sinuses do not have traversing reticular fibres and macrophages, but lymphocytes occur in them. In the upper end of the MLN lipid (L) is in the sinus. (e) An electron micrograph of a follicular dendritic cell.

can gain access to the dense lymphatic tissue of the MLN and react with it (Good and Finstad, 1967; McCorkle *et al*., 1979; Oláh and Glick, 1983). After injection of sheep red blood cells, plaque-forming cells appear in the spleen suggesting that activated lymphoid and/or accessory cells leave the MLN and enter the blood circulation (McCorkle *et al*., 1979).

Lymphoid tissue of the large MLN does not differ appreciably from that of a "true" lymph node. GC are embedded into dense lymphoid tissue occupied by B and T cells. Although the number of GC in the MLN is low, the proportion of B cells is higher than T cells, whereas the converse applies in the "true" lymph node. This finding suggests that the interfollicular region, which corresponds to the paracortex of the "true" lymph node, is not well-defined in birds and contains a large number of B cells besides T cells (McCorkle *et al*., 1979). There is no HEV in the MLN, but lymphocyte migration frequently occurs through the wall of the sinuses (Oláh and Glick, 1983). The size of the MLN seems to be related with its histological structures, namely the presence of sinuses and GC, allowing classification of MLN in three different groups. Possibly these three groups represent different developmental stages rather than different structural identities. Type I is the smallest consisting of only a lymphoid accumulation on one side of the lymphatic without sinuses or GC (Fig. 2.9(a) and (b)). These nodes are not encapsulated, tightly surrounded by adipocytes, and blood vessels may penetrate them. The second type is basically an enlarged type I with few GC. The type II node frequently contains many scattered erythrocytes (Fig. 2.9(c)) and occasionally, small, possibly blind-ended, out-pocketing of the lymphatic may also occur. The third and largest MLN produces branching sinuses and many GC embedded into the dense lymphoid tissue (Fig. 2.9(d)). The second and third types of nodes are partially encapsulated, located between the lymphatic and a surrounding loose connective-tissue sheath. The space between the lymphatic and the sheath is filled with fat and lymphoid tissue.

MLN GC are identical to others located elsewhere in the peripheral lymphoid tissue (Fig. 2.9(c) and (d)). GC can be divided into a peripheral zone, which does not have FDC, although the lymphoid cells synthesize DNA and are mitotically active. The central zone contains FDC with lymphoid cells that are less mitotically active. The surface of the FDC is highly indented and covered by an electron-dense substance, which possibly consists of immune complexes (Fig. 2.9(e)). The cytoplasm reveals several electron-dense granules in different shapes and sizes intermingled with well-developed cytoplasmic organelles.

The histology of MLN indicates that their formation takes place in two ways: either outside the wall of the lymphatics (Fig. 2.9(a)–(d)) or inside the lumen of the lymphatics. The former does not narrow the lumen, because the cells migrate out of the lymphatic and settle in the compartmentalized adipose tissue, while the latter is formed inside the lymphatic. In the distended lymphatic the endothelium becomes adhesive for lymphoid cells. These lymphoid cells become covered by newly formed endothelial cells or migrate through it (Oláh and Glick, 1985). This process is repeated several times resulting in a lamellated structure inside the lymphatic. The sinuses in this MLN are formed from the original lumen of the lymphatic, from where the lymphocytes continuously migrate through the newly formed endothelium enlarging the MLN.

ECTOPIC LYMPHOID TISSUE AND THE PINEAL GLAND

Ectopic lymphoid tissue begins to develop after 3-week of age in non-lymphoid organs such as liver, pancreas, kidney, endocrine glands (thyroid, adrenal and pituitary), gonads and even central nervous system brain and spinal cord (Payne, 1971; Hodges, 1974). A major unresolved question is whether ectopic lymphoid tissue represents a burst of lymphomatosis, which is destructive for the non-lymphoid organ, or a normal reaction to external antigens. Biggs (1957) considered the appearance of the ectopic lymphoid tissue a normal reaction of the avian immune system. Ectopic lymphoid tissue is transient – disappearing at about 3–4 months of age when the non-lymphoid organ returns to its intact state – both histologically and functionally. The

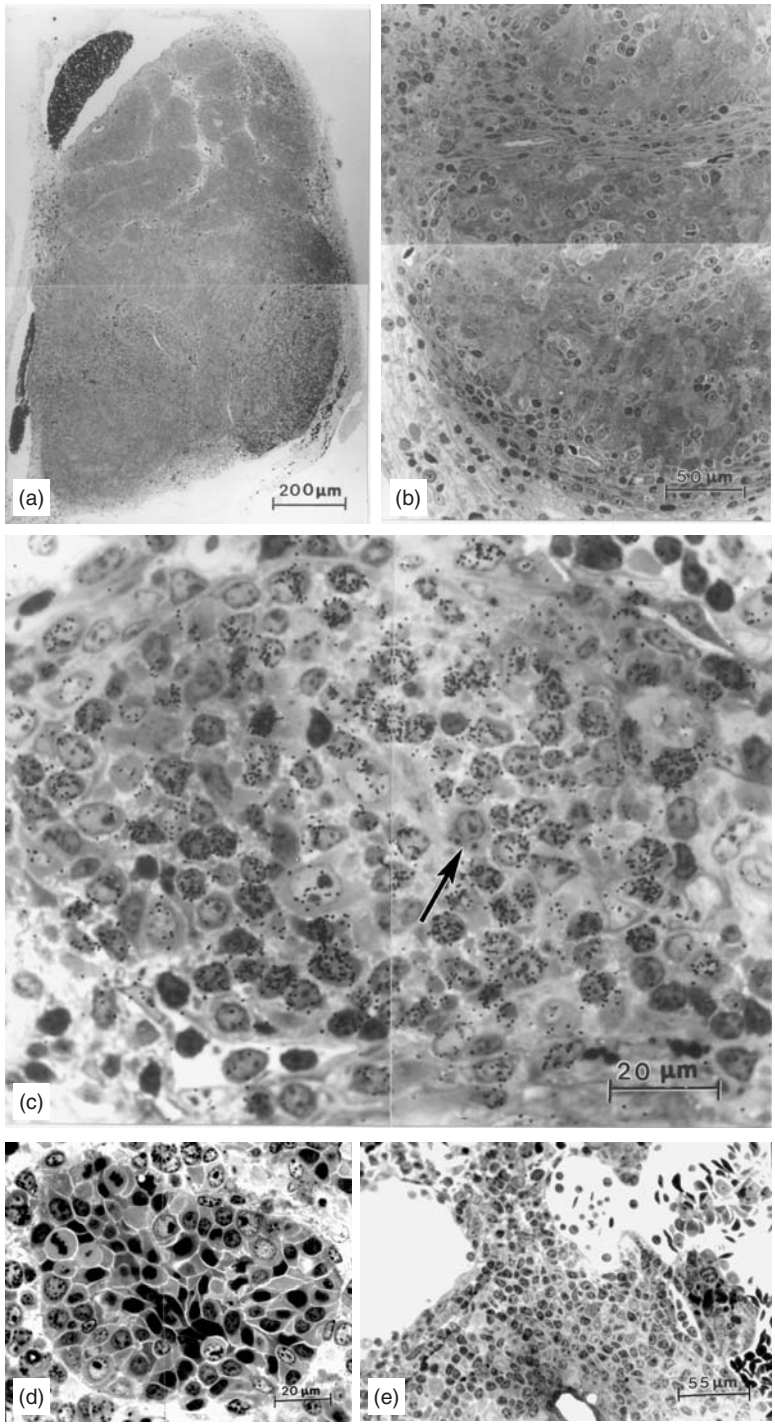

FIGURE 2.10 Details of the pineal lymphoid tissue. (a) The upper part of the gland shows pineal vesicles with a slit-like lumen, while the lower part is infiltrated with lymphoid tissue. (b) A portion of a pineal vesicle is surrounded by lymphoid cells in the pial septae. Many lymphoid cells invade the wall of the pineal vesicle creating the lymphopineal tissue. (c) In the germinal centre the majority of the cells are in S phase of cell cycle, which is indicated by ^{3}H-thymidine uptake. The follicular dendritic cell is not labelled (arrow). (d) Several parts of the bone marrow sinuses are densely packed with erythroid and perhaps thrombocytic cells. Immature cells and mitotic figures are attached to the endothelial cells, while more mature cells are located near the centre of the sinus. (e) Lymphopoiesis takes place in foci around the arteries of the bone marrow.

possibility that ectopic lymphatic tissue destroys the normal organ, needs to be reconsidered. In chickens there is a lymphoid burst after 3 weeks of age, which is manifested by the appearance of lymphoid tissue in non-lymphoid organs. In recent years, it has been shown that stem cells occur in almost every adult tissue, and it is not surprising that lymphoid foci (more or less organized lymphoid tissue) appear in many non-lymphoid organs causing no major functional disturbances. One such ectopic lymphoid tissue emerges in the pineal gland, an organ usually beyond the scope of avian immunologists.

Lymphoid infiltration in the pineal gland begins around 3 weeks of age and may increase to 50% of the pineal mass (Cogburn and Glick, 1981). This lymphoid tissue is immunologically active and capable of antibody production (Cogburn and Glick, 1983). The lymphoid accumulation is closely associated with the vein at the distal end of the gland (Fig. 2.10(a)) and fills up the pial or pineal septae (Romieu and Jullien, 1942), where GC are formed. The pineal parenchyma is isolated from the pial connective tissue by a BL of the pinealocytes, through which mobile lymphatic and myeloid cells enter the wall of the pineal vesicles (Fig. 2.10(b)). The topographical relationship between the cells of the pineal gland and lymphoid cells is identical to that in the lymphoepithelial tissue (Oláh and Glick, 1983). From the wall of the pineal vesicles, the lymphoid cells can enter the lumen of the vesicles. The invading cells are lymphocytes, plasma cells, and a few myeloid cells, mainly eosinophil and basophil granulocytes. Thymidine uptake by lymphocytes located inside the pineal parenchyma is about twice as high as that of lymphocytes in the pial connective tissue. This high rate of lymphocyte proliferation (Fig. 2.10(c)) suggests that the pineal produces some kind of cytokine (Oláh and Glick, 1984). In the lymphopineal tissue there is an intimate connection between pinealocytes and lymphoid cells, producing a unique interaction between the immune and central nervous systems. Lymphoid tissue in the pineal is organized like its peripheral counterpart into dense lymphoid tissue and GC that contain B and T cells, respectively.

BONE MARROW

Histologically, two separate compartments can be distinguished in the bone marrow. The intravascular compartment is, possibly, responsible for the erythro- and thrombopoiesis, while the extravascular compartment is responsible for myelo-, mono- and lymphopoiesis. After hatching many bone marrow sinuses appear densely packed with immature cells of the erythroid lineage. Thrombocytes cannot be distinguished on histological sections. The sinus endothelial cells are flat – and frequently seem to be discontinuous – allowing cell migration between the two compartments. Most immature cells are located close to the sinus endothelium, while the mature red blood cells generally occupy the centre of the sinus. Mitosis is common, indicating that the sinus – unlike in mammals – contains an actively proliferating cell population, which are possibly erythroid progenitors. In some cases the sinus appears so crowded with immature cells that circulation appears to be very slow or to have stopped. This provides a transitory, niche and a haematopoietic microenvironment for the erythroid and/or thrombocytoid cell maturation. Granulocytes inside the sinuses can only be found occasionally.

The extravascular compartment is filled with granulocytes in different stages of maturation, and a few lipid cells (Fig. 2.10(d)). With increasing age the granulocytopoiesis becomes restricted to small areas in the extravascular compartment, while the majority of this place contains only few scattered cells among the increasing number of adipocytes. The intravascular compartment contains mature erythrocytes and occasional immature blast-like cells. In the extravascular compartment the lymphopoiesis occurs in close proximity of the bone marrow arteries. Unlike granulopoiesis, which is scattered throughout the bone marrow, lymphopoiesis is restricted in small foci (Fig. 2.10(e)).

The crude supporting system of the bone marrow is formed by three-dimensional branching, bony spicules whose surfaces may be covered by giant, multinucleated osteoclasts. Their free

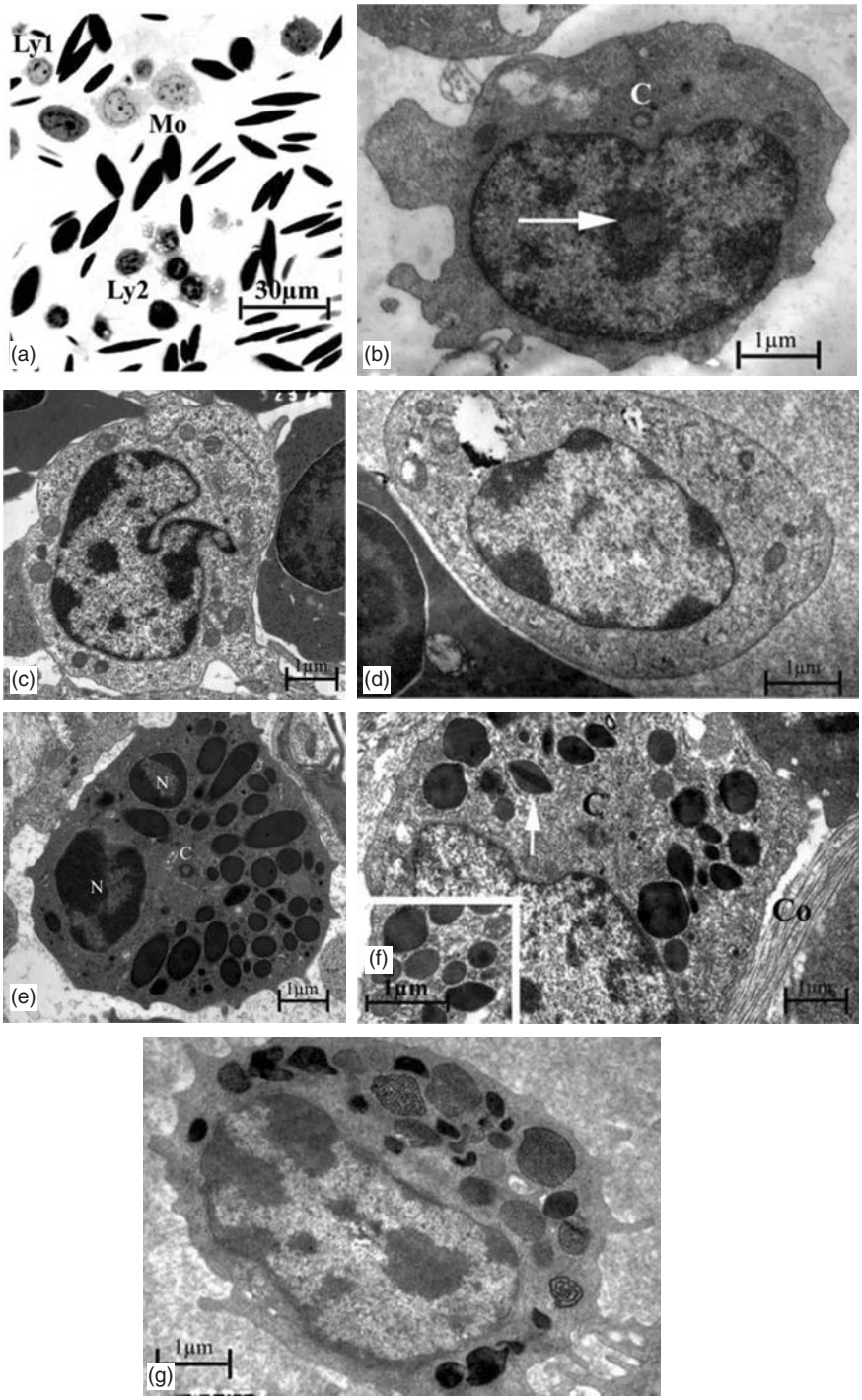

FIGURE 2.11 (a) A light micrograph showing three monocytes (Mo), medium-sized (Ly1) and small (Ly2) lymphocytes with highly ruffled surface membranes. Ovoid-shaped erythrocytes are shown in different planes. (b) *Lymphocyte*: In the centre of the nucleus the heterochromatic substance is associated with the nucleolus (arrow). The centriole (C) is located close to the moderate indentation of the nucleus. The cytoplasm contains only few mitochondria. (c) *Monocyte*: The nuclear membrane is highly invaginated and the cytoplasm rich in organelles: mitochondria, free ribosomes, smooth- and rough-surfaced endoplasmic reticulum and few granules. (d) *Thrombocyte*: Ovoid-shaped cell reveals a thick layer of cytoplasm with few mitochondria and vesicles. (e) *Heterophil granulocyte*: Two lobes of the nucleus (N) are evident in this section. The centriole (C) is surrounded by round- and ovoid-shaped variably sized granules. (f) Eosinophil granulocyte in the connective tissue. The eosinophil-specific granule contains a crystal in its centre (arrow), centriole (C), collagen fibres (Co). Inset: two of the specific granules reveal crystals, while the content of the azurophilic granules is more heavily electron dense and homogeneous. (g) *Basophil granulocyte*: Around the kidney-shaped nucleus irregularly shaped and sized, highly textured granules fill the cytoplasm.

surface is ruffled and monocyte-like cells frequently occur alongside. These osteoclasts together with the sinus endothelium, bone marrow reticular cells and adipocytes are the major stromal cells providing the haematopoietic microenvironment for the pluripotent haematopoietic stem cells.

The haematopoietic stem cells can differentiate to form common myeloid and lymphoid progenitors by asymmetrical cell division. One of the daughter cells remains in the haematopoietic stem cells pool, while the other differentiates to form one of the progenitor cells. In mammals erythropoiesis takes place in the extravascular compartment and it is generally accepted, that the common myeloid progenitors provide the common megakaryocyte and erythroid progenitors. In chicken thrombopoiesis and erythropoiesis occur in the sinuses, consequently some sinuses, or parts of thereof, could provide a haematopoietic microenvironment by temporarily slowing down, or even stopping, the circulation.

BLOOD

Blood can be considered a special type of connective tissue, in which the extracellular matrix is fluid: the plasma in which blood cells are suspended. There is a continuous exchange of products and cells between the plasma and the extravascular tissue fluid. Plasma serves as a vehicle for gases, nutrients, hormones, proteins, such as albumin and Ig, fibrinogen and metabolic waste products. The cellular content, on the basis of function, consists of three major cell types: the erythrocytes, the leukocyte lineage and the thrombocytes – involved, respectively, in the transport of oxygen and carbon dioxide; immune and inflammatory reactions and phagocytosis and the control of bleeding. Leukocytes are present in the blood only transiently (generally 12–20 h). After this relatively short period they leave the circulation and migrate into the tissues, where they perform their specialist functions. Detailed descriptions of the blood cells and plasma are beyond the scope of this chapter; however, leukocytes are such important components of the immune system that the basic cytological structure of the key players is shown in Fig. 2.11.

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3

DEVELOPMENT OF THE AVIAN IMMUNE SYSTEM

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INTRODUCTION
ORIGINS AND MIGRATION ROUTES OF HAEMATOPOIETIC CELLS USING QUAIL/CHICK COMPLEMENTARY CHIMAERAS
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INTRODUCTION

The avian embryo provides several advantages for studies on the development of the immune system. These include the existence of a clear demarcation between the B and T cell systems, with each population differentiating in a specialized primary lymphoid organ – T cells in the thymus and B cell in the bursa of Fabricius. In addition, there is an availability of large numbers of embryos at precise stages of development. Because of their importance to the poultry industry, much research on the avian system has used the domestic chicken and this has been helped by the ready availability of different congenic and inbred lines, genetic markers and monoclonal antibodies (mAb). These provide essential tools for studying the development of the immune system. The chick–quail chimaeras have also proved to be an especially informative model, particularly for studying the emergence of haematopoietic stem cells (HSC) and their migration to the primary lymphoid organs during embryogenesis.

ORIGINS AND MIGRATION ROUTES OF HAEMATOPOIETIC CELLS USING QUAIL/CHICK COMPLEMENTARY CHIMAERAS

Looking for the Source of Haematopoietic Cells During Development

Based on the pioneering avian studies almost a century ago, it was proposed that the yolk sac must be the site which gives rise to all haematopoietic cells (HC). This was proposed because

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the first blood cells, i.e. the primitive erythrocytes, appear in this embryonic appendage (Dantschakoff, 1907; Maximow, 1909). Later experiments, using parabiosed avian embryos, led Moore and Owen (1965, 1967a, b) to expand on this view and propose that definitive HSC are generated only once, early in embryonic development and migrate from the yolk sac to seed the haematopoietic organ rudiments; bone marrow being the site of life-long haematopoiesis. Early experiments with mouse embryos supported this view (Moore and Metcalf, 1970; Weissman *et al*., 1978). Soon afterwards, taking advantage of a newly discovered quail/chick marker (Le Douarin, 1969), a series of elegant grafting experiments was performed by Dieterlen-Lièvre and co-workers who challenged the notion of the yolk sac origin for definitive HSC. They used a unique type of quail/chick chimaera, initially devised by Martin (1972), which consisted of homotypic grafting of a quail embryo onto a chick yolk sac at a very early stage in development, before the onset of heart beating. Such "complementary chimaeras" revealed that the cells colonizing the haematopoietic organs were derived from the embryo and not the yolk sac (Dieterlen-Lièvre, 1975; Martin *et al*., 1978; Beaupain *et al*., 1979). The yolk sac did generate definitive blood cells, but their numbers decreased rapidly. Circulating blood cells were of chick origin (derived from yolk sac progenitors) until embryonic incubation day (EID) 5 when they became mixed and, subsequently, increasingly rich in quail erythrocytes (Beaupain *et al*., 1979). This replacement was confirmed when chimaeras were constructed between congenic strains of chickens differing in their immunoglobulin (Ig) allotypes or major histocompatibility complex (MHC) antigens (Lassila *et al*., 1978, 1982). Therefore, in contrast to the earlier hypothesis, these results led to the undisputable conclusion that yolk-sac-derived progenitors contributing to a single transient wave of haematopoiesis, whereas it is the embryo proper contributes the definitive HSC that maintain life-long haematopoiesis.

Macrophage Production by the Yolk Sac

In addition to producing erythrocytes, another function of the yolk sac is to produce the first generation of macrophages. Using reversed yolk sac chimaeras (chick embryos onto a quail yolk sac) combined with cell-type- and species-specific mAb, such as QH1 and MB1 (Péault *et al*., 1983; Pardanaud *et al*., 1987) that recognize quail HSC and endothelial cells, respectively, it was demonstrated that a small HC population of yolk sac origin differentiates into primitive macrophages. These are located in zones of apoptosis, notably in neural derivatives, and may represent the first microglial cells (Cuadros *et al*., 1992; Cuadros and Navascues, 2001). Such a contribution from the yolk sac to the early macrophage population has also been demonstrated for the mouse embryo (Alliot *et al*., 1999; Bertrand *et al*., 2005), confirming that mechanisms giving rise to the different haematopoietic lineages are strongly conserved among species.

The Aortic Region Produces HSC

Once it had become abundantly clear that another source must provide the definitive HSC, the aortic region became the prime candidate for their origin. Much earlier, the aorta had been shown to contain clusters of putative HC protruding from the ventral endothelium (Emmel, 1916; Jordan, 1917, 1918). Cells in these clusters exhibited a high nuclear-to-cytoplasmic ratio and an affinity for basophilic stains, both indicative of a haematopoietic nature (Dieterlen-Lièvre and Martin, 1981), and were later shown to express haematopoietic markers (Pardanaud *et al*., 1987; Jaffredo *et al*., 1998). Subsequent *in vitro* and *in vivo* functional analyses demonstrated that the definitive haematopoietic system originates in this aortic region (Lassila *et al*., 1980; Cormier *et al*., 1986; Cormier and Dieterlen-Lièvre, 1988; Cormier, 1993). Whether these haematopoietic clusters were the source of HC was not clear from these experiments, because the avian embryonic aortic region also contains para-aortic foci; large groups of cells ventral to the aorta which emerge after the clusters have been formed (Sabin, 1917; Dieterlen-Lièvre and Martin, 1981; Jaffredo *et al*., 2000), and which have been shown to harbour HSC (Toivanen *et al*., 1979; Plate 3.1).

AORTIC CLUSTERS AS THE INTRA-EMBRYONIC SOURCE OF DEFINITIVE HAEMATOPOIESIS

Cellular and Molecular Identification of the Clusters

The most prominent feature of aortic haematopoiesis is the presence of small groups of HC protruding into the aortic lumen, the intra-aortic clusters. Intra-aortic clusters are invariably restricted to the ventral aspect or floor of the aortic endothelium (as illustrated in Plate 3.2), but they extend to the vitelline arteries where, importantly, they display the same ventral polarization. The distribution is identical to that described for mouse and human embryos (Garcia-Porrero *et al*., 1995; Tavian *et al*., 1996; Wood *et al*., 1997; Marshall *et al*., 1999; de Bruijn *et al*., 2002). The clusters appear soon after the two distinct, original aortic anlagen fuse to form the dorsal aorta. The haematopoietic nature of cells in the clusters is confirmed by the expression of the pan-leukocytic antigen CD45 (Plate 3.2(b); Jaffredo *et al*., 1998, 2000). Furthermore, these cells exhibit the CD41 antigen or αIIb integrin subunit (Dieterlen-Lièvre and Le Douarin, 2004). This integrin, which is the fibrinogen receptor, was first recognized for its important functional role in megakaryocytes and platelets (Naik and Parise, 1997). It was later found on the surface of myeloid progenitors and finally adopted as a diagnostic tool for HSC, on the basis of its marked expression by the intra-aortic clusters in the chicken (Ody *et al*., 1999) and in the mouse (Corbel and Salaun, 2002). CD41 is now used as the hallmark of HSC in the foetus (Emambokus and Frampton, 2003; Mikkola *et al*., 2003). Interestingly, the endothelial-specific αv integrin subunit (CD51) and the haematopoietic-specific CD41 display complementary patterns in the dorsal aorta (Plates 3.2(a) and (c)).

On the whole, cluster cells share most of the surface proteins with endothelial cells and express many common transcription factors. Several molecular features other than CD41 make it possible to distinguish the clusters from the endothelium. The haematopoietic clusters are sharply distinguished from endothelial cells by the expression of transcription factors c-myb and Runx1, the pan-leukocytic CD45 antigen and by the disappearance of VEGF-R2 and VE-cadherin (Jaffredo *et al*., 1998, 2005; Bollerot *et al*., 2005).

The Para-Aortic Foci

A later aspect of aorta-associated haematopoiesis, prominent between EID 6 and 9, is the presence of large groups of HC located in the loose mesenchyme, ventral to the aorta. Due to their position, these structures have been designated as the para-aortic foci. These cells were shown to be responsible for the first wave of thymus colonization (Dunon *et al*., 1998, 1999) and, at the same time, seeding of the bone marrow (Le Douarin *et al*., 1984). Whereas a few cells expressing HC characteristics have been reported as present in a similar location in the mouse, the existence of a homologous site has not yet been confirmed in mammals.

Tracing the Origins and Fates of the Aortic Clusters

The long-proposed hypothesis that, endothelial cells from the aorta give rise to HC, was investigated in the avian model *in vivo*. The fate of aortic endothelial cells was followed by tagging the aortic endothelium with two vital tracers, either acetylated low-density lipoprotein (AcLDL) coupled with a fluorescent lipophilic marker (DiI) or a non-replicative retroviral vector carrying a reporter gene. The first labelling method has two major advantages: (1) the tag is endocytosed *via* a specific receptor on endothelial cells and macrophages (the latter are absent at the stage of inoculation) and (2) it has a short half-life and quickly disappears from the circulation, if it is not immediately endocytosed. The second method allows stable expression of the vector's reporter gene for an extended period of time and allows the identification of clones since integration occurs in sparse cell populations. Both tracers are inoculated into the heart, ensuring direct contact with the endothelium lining the vessels, early on EID 2, 1 day before the emergence of the aorta-associated HC. Cell identification can be achieved using the anti-VEGF-R2 (Eichmann *et al*., 1997) and anti-CD45 antibodies (Jeurissen and Janse, 1998) as endothelial cells and HC probes, respectively.

As early as 2 h after AcLDL-DiI inoculation, the whole vascular tree is labelled. The aortae, still paired at this time, are entirely lined by AcLDL-DiI$^+$ endothelial cells, while no CD45$^+$ cells are present in the mesenchyme around the aorta or in the circulation. One day after inoculation, when haematopoietic clusters emerge, all cells therein are AcLDL-DiI$^+$/CD45$^+$. Retrovirus-labelled clones, on the other hand, comprise endothelial cells or HC but never both (Jaffredo *et al.*, 2000).

AcLDL-DiI$^+$/CD45$^+$ double positive cells are also found in the mesenchyme ventral to the aorta suggesting that a subset of HC have ingressed into this region from the ventral endothelium (Jaffredo *et al.*, 1998). Moreover, after retroviral labelling, the haematopoietic foci, which develop in the mesenchyme ventral to the aorta, contain numerous lacZ$^+$ cells (Jaffredo *et al.*, 2000). Thus, these foci are seeded by progenitors derived from the aortic floor at EID 3.

We therefore conclude that in the avian embryo during the first week of development, intraembryonic progenitors are derived from the aortic floor through a unique process that switches the fate of cells which previously contributed to the endothelial lining of the aorta. In order to elucidate the origin and peculiar cell disposition of the clusters, it is necessary to trace back the developmental history of the aorta.

FORMATION OF THE AORTA: A DORSAL ANGIOBLASTIC LINEAGE AND A VENTRAL HAEMANGIOBLASTIC LINEAGE

Two Endothelial Lineages form the Vascular Network of the Embryo

The embryonic mesoderm, i.e. the germ layer which gives rise – together with other derivatives – to the blood system, is laid down during gastrulation as a single sheet; it then divides into several medio-lateral components, whose respective contributions to the blood/vascular system were established in a series of experiments employing the quail/chick system. The conclusions were as follows: the somatopleural derivatives (limb buds and body wall) are vascularized by exogenous angioblasts (Pardanaud *et al.*, 1989), by a colonization process known as angiogenesis (Risau and Lemmon, 1988); while viscera (splanchnopleural derivatives) form their vascular network from their own mesoderm, a process designated vasculogenesis (Pardanaud *et al.*, 1989). The source for endothelial cells colonizing the somatopleural derivative was later shown to be the somite (Pardanaud *et al.*, 1996). This dual mode of blood vessel development has been confirmed recently using the mouse model (Pudliszewski and Pardanaud, 2005).

Chimaeric Origin of the Aortic Endothelial Cells

Interestingly, the experiments of Pardanaud *et al.* (1996) have also led to the conclusion that somite- and splanchnopleura-derived angioblasts have distinct homing potentials in the aorta. The somite-derived angioblasts integrate into the roof and sides of the aortic endothelium. Importantly, they never penetrate visceral organs or integrate into the aortic floor. In contrast, angioblasts derived from splanchnopleural mesoderm invade the floor of the aorta and visceral organs. Furthermore, when integrated in the aortic floor – and only there – cells of splanchnopleural origin proliferate into clusters. Thus, the homing site is a second requirement for the emergence of clusters. To summarize, the body wall (somatopleura), unable by itself to make blood vessels, becomes vascularized by angioblasts emigrating from the somites, while the splanchnopleural mesoderm gives rise to both angioblasts and HSC, which may be derived from a putative common progenitor, the aortic haemangioblast. The name aortic angioblast was first coined by Murray (1932) and describes the homogeneous cell aggregates that precede yolk sac blood islands (Murray, 1932). In its modern meaning, this term defines a putative common progenitor of angioblasts and HSC.

The mosaic appearance of the aortic floor at the peak of cluster formation (on EID 3, 24 h after grafting) was described in studies conducted by Pardanaud *et al.* (1996). Recently, one of

us obtained a dynamic image of aortic evolution using a modified version of the same experimental approach, though differing in two crucial aspects (Pouget *et al*., 2006). Firstly, the grafted material involved the whole segmental plate (approximately 10 future somites) plus the last segmented somite; secondly, the host embryos were examined at different time points (Plate 3.3). Quail somitic cells invaded the roof of the chick aorta around 15 h after grafting. At 24 h, only the aortic floor, as well as the clusters, were from the chick host (i.e. splanchnopleural). When the clusters disappeared, the floor became quail (i.e. derived from the grafted somitic material). In other words, the cluster-forming splanchnopleural cells disappear after HC production. As a result, the endothelium of the definitive aorta at EID 4–4.5 is entirely composed of somite-derived cells. The inability of somitic angioblasts to penetrate the viscera was confirmed using this modified method, despite the replacement of much longer strips of somitic material.

These experiments shed an interesting light on the problem of developmental relationships between endothelium and HSC, showing that the aortic floor endothelium is a transient structure which becomes spent and replaced as haematopoiesis terminates. In addition, they provide an explanation for the brevity of intra-aortic cluster production, a feature observed in all the species studied so far; haematopoietic production in this site depends on a limited, non-renewable pool of haematogenic endothelial cells that further give rise to the para-aortic foci (Plate 3.3 right side).

The Allantois: Another Source of Haematopoiesis?

In birds, the timing of the production of intra-aortic clusters and para-aortic foci is compatible with the seeding of the thymus and bursa of Fabricius. Cells of the para-aortic foci have been shown to harbour multipotent progenitors, with HSC probably among them. However, seeding of the bone marrow begins by EID 10.5 in the chick embryo and continues until hatching. At this time, the activity of the para-aortic foci has terminated. The possibility that cells from the para-aortic foci seed the bone marrow during its very late phases of colonization appears unlikely. Therefore, another progenitor-producing site has been sought and identified as the allantois. This appendage has been shown to be involved in gas exchange, excretion, shell Ca^{++} resorption and bone formation. It also has the appropriate tissue make-up for haematopoiesis, i.e. endoderm associated with mesoderm.

Cellular and Molecular Identification of Allantois-Associated Haematopoiesis

By using Indian ink or AcLDL-DiI microangiographies, we have established that the allantois becomes vascularized at between 75 and 80 h of incubation. It displays conspicuous red cells even before vascularization, indicating that haematopoiesis occurs at this site quite independently of the rest of the embryo (Caprioli *et al*., 1998, 2001). The allantois follows a programme of development characterized by the prominent expression of several "haemangioblastic" genes in the mesoderm and of other genes in the associated endoderm. VEGF-R2 is expressed in the mesoderm, at least from stage HH17 onward. Shortly afterwards it is followed by the expression of several transcription factors (GATA-1, GATA-2 and SCL/tal-1). Blood island-like structures which contain both $CD45^{+}$ cells and cells which accumulate haemoglobin differentiate. These structures look exactly like the blood islands in the yolk sac. This haematopoietic process takes place before the vascular network connecting the allantois to the embryo is established. As far as the endoderm is concerned, GATA-3 messenger (m)RNA is found in the region where the allantois differentiates before the posterior intestinal portal becomes anatomically distinct. Shortly before the allantoic bud grows out, GATA-2 is expressed in the endoderm and, at the same time, the haemangioblastic programme is initiated in the mesoderm. GATA-3 is detected at least until EID 8 and GATA-2 until EID 3, the latest times examined for these factors. Using *in vitro* culture techniques we have shown that those allantoic buds, dissected out before the circulation between the bud and the rest of the embryo is established, produce elliptical erythrocytes, typical of the definitive lineage. Moreover, using heterospecific grafts between chick and quail embryos, we were able to demonstrate that the allantoic vascular network develops from

intrinsic progenitors (Caprioli *et al*., 2001). Taken together, these results extend earlier findings concerning the commitment of mesoderm to endothelial and haematopoietic lineages in allantois. Detection of prominent GATA-3 expression, restricted to the endoderm of the pre-allantoic region and allantoic bud, followed by that of GATA-2 expression is novel in the context of organ formation and endoderm specification with respect to the emergence of HC.

Haematopoietic Production by the Mammalian Allantois and the Placenta

Interestingly, it has recently been demonstrated that the mouse allantois and its further derivative, the placenta, are present at the site of haematopoietic emergence (Zeigler *et al*., 2006; Corbel *et al*., 2007) and HSC amplification (Gekas *et al*., 2005; Ottersbach and Dzierzak, 2005), respectively. Taken together, these data strongly suggest a previously unappreciated role for the placenta as a source of HSC. Further studies are needed to determine the origins of placental HSC and the characteristics the placental microenvironment provides to support HSC activity.

THE AVIAN THYMUS AND T CELL DEVELOPMENT

Thymic Development

Avian T cell development has been found to be remarkably similar to that of mammals, although it has some unique features. Using quail–chick chimaeras, it was shown that the thymus becomes populated with cells originating in the three germ layers (i.e. ectoderm, mesoderm and endoderm). Stromal epithelial cells form a network, which contains abundant lymphoid cells in both cortex and medulla. These stromal cells originate from endodermal buds arising from the 3rd and 4th branchial pouches (for further details, see Chapter 2). The connective tissue, which separates the thymic lobes and lobules, and the pericytes and smooth muscle cells associated with intra-thymic blood vessels are derived from the ectoderm via the neural crest. The haematopoietic component, which differentiates in the thymus into T lymphocytes, macrophages and medullary dendritic cells, is derived from the mesoderm. The endothelial network originates from the mesoderm (Le Douarin and Jotereau, 1975; Couly *et al*., 1995). The mesenchymal cells which participate in thymic histogenesis are critical for the differentiation of the endodermal thymic stroma. As shown using quail–chick chimaeras, in the head of the vertebrate embryo there are supporting tissues (bone cartilage, connective cells but not striated muscles), which originate from a unique type of mesenchymal cells, that originate in the neural crest, the so-called mesectoderm.

Mesenchymal cells are closely associated with the endodermal thymic rudiment which, in chick and quail embryos, first appears on EID 3 on either side of the pharynx as two buds from the 3rd and 4th pharyngeal pouches (in the mouse, a unique thymic bud arises from the 3rd pharyngeal pouch). One day later, the thymic rudiments have detached from the pharynx and form two compact cords of epithelial cells which, eventually, fuse and become surrounded by blood vessels. Further development of the thymic stroma depends on signals arising from the surrounding mesenchymal cells. These signals are responsible for the rapid transformation of the thymic epithelia into a lymphoid organ. The well-defined cortex and medulla are established by EID 12 (Coltey *et al*., 1987).

Colonization of the Thymus

That all lymphocytes developing in the thymus are of extrinsic origin was demonstrated by experiments in which the thymic rudiment was removed at regular intervals from EID 3 to EID 7 quail or chick embryos and grafted into the somatopleure of the other species. It was shown that the thymic epithelium becomes attractive to blood-borne HC at precise stages in development. T cell progenitors colonize the epithelial thymus in three successive waves, the first beginning at EID 6, the second at EID 12 and the third occurring around EID 18 until just

after hatching (Coltey *et al*., 1989; Plate 3.1). Each wave of thymic colonization lasts for 1 or 2 days and is separated from later waves of progenitor cell influx by refractory periods. Although no information about the internal clock governing thymic influx is available, it has been demonstrated that each wave of colonization is correlated with a peak in the number of progenitor T cells present in the blood circulation (Dunon *et al*., 1999). Progenitor cells are almost completely absent from the blood during the refractory periods. Experiments in which T cell progenitors have been injected intravenously have clearly shown that these cells are able to home to the thymus without delay, even during the refractory periods (Dunon *et al*., 1999). Adoptive transfers have shown that T cell progenitors in the first wave originate from para-aortic haematopoietic foci, whereas progenitors in the second and third waves originate from the bone marrow (Cormier, 1993; Vainio and Imhof, 1995; Dunon *et al*., 1999). Some molecules expressed on the surface of the bone marrow T cell progenitor have been identified, these include c-kit, HEMCAM, MHC class II antigens and αIIβ3integrin (Ody *et al*., 2000). Interestingly, expression of the terminal deoxynucleotidyl transferase (TdT) appears on thymocytes at EID 12, i.e. after the first wave, and increases linearly thereafter suggesting that the second generation of lymphocytes becomes TdT positive (Bollum, 1979; Penit *et al*., 1985). TdT is the crucial enzyme involved in the non-germline nucleotide addition (N-region) during V(D)J T cell receptor (TCR) gene segment recombination and can be regarded as an early T cell marker (Desiderio *et al*., 1984). Expression of TdT appears at a critical developmental stage for the thymic lymphocytes; namely when they begin to be immunologically competent.

T Cell Differentiation

Progenitors in each wave give rise to γδT cells about 3 days earlier than the appearance of αβT cells. The development of each T cell lineage can be traced using mAb recognizing different chains of TCR. At EID 12, about 5 days after the initial influx of thymocyte precursors, a subpopulation of thymocytes begins to express the γδTCR-CD3 complex on the surface (recognized by the TCR1 mAb; Sowder *et al*., 1988). Numbers of $TCR1^+$ cells increase reaching a peak by EID 15, when about 30% of the thymocytes express the γδTCR. Cells that express the γδTCR-CD3 complex fail to express either CD4 or CD8 accessory molecules. αβTCR-bearing T cells which express the Vβ1 variable domain (recognized by the TCR2 mAb) are first detected in the thymocyte population on EID15, and these become the predominant type of thymocytes by EID 17–18 (Coltey *et al*., 1987). αβTCR-bearing cells expressing the Vβ2 variable domain (recognized by the mAb TCR3) emerge around EID 18. The developmental patterns of $TCR2^+$ cells and $TCR3^+$ cells appear to be very similar to those described for mammalian αβT cells, where there is dual expression of the CD4 and CD8 accessory molecules on the surface of both $TCR2^+$ and $TCR3^+$ cortical thymocytes (Coltey *et al*., 1989). After expansion in the cortex, these $CD4^+CD8^+$ double-positive cells undergo clonal selection and maturation to become either single positive $CD4^+$ or $CD8^+$ T cells (Davidson and Boyd, 1992).

TCR Rearrangement

Genes encoding the α, β, γ and δ chains of the chicken TCR have been cloned (Litman *et al*., 1999). The TCRβ locus contains two Vβ gene families, 14 Dβ gene segments, 4 Jβ elements and a single Cβ gene. The two Vβ families contain six and four Vβ elements, respectively. TCRβ rearrangements involving gene segments from the Vβ1 gene family can be detected beginning on EID 12, while rearrangements involving the Vβ2 family are first detected on EID 14 (Pickel *et al*., 1993). As with mammals, the chicken TCRδ gene locus is nested within the TCRα locus. Several Vα and multiple Jα elements are associated with a single Cα region. The large number of Jα elements could favour successive rearrangements of the TCRα locus and therefore enhance the production of in-frame rearranged TCRα chains. One Vδ gene family (20–30 Vδ elements), two Dδ gene segments, two Jδ gene segments and one Cδ gene have been identified. Vα and Vδ gene segments rearrange with one, both or neither of the Dδ segments, and with either of the

two Jδ segments. Three different Vγ families, each with about 10 members, three Jγ elements and a single Cγ region have been identified in the chicken (Six *et al*., 1996). Random V, (D), J rearrangements, exonuclease activity, template (P) and non-template (N) nucleotide addition contribute to the diversification of the TCR repertoire during avian T cell differentiation. This process is described in detail in Chapter 5.

T Cell Homing to the Periphery

Chick–quail chimaeras, congenic chicken strains and mAb have been used successfully to trace T cell migration patterns during ontogeny (Dunon *et al*., 1997). Both TCRγδ ($TCR1^+$) and TCRαβ $V\beta_1$ ($TCR2^+$) thymocyte populations migrate to the periphery in the same order as their appearance in the thymus. The γδT cells constitute the major population of lymphocytes in epithelial-rich tissues where they act as the first line of defence against invading pathogens. In contrast to humans and mice, where the γδT cells represent only about 5–10% of peripheral lymphocytes, chickens have a much larger (20–60%) population of these cells; the difference in the frequency between species has led to a demarcation between γδ-low and γδ-high species, the chicken being a member of the latter (Kubota *et al*., 1999). In the spleen γδT cells appear at EID 15 whereas the TCRαβ $V\beta_1$ ($TCR2^+$) do not reach the spleen until EID 19. TCRαβ $V\beta_2$ ($TCR3^+$) cells are not found in the spleen until the second day after hatching (Cooper *et al*., 1989). These three T cell populations display distinct homing patterns in the spleen: γδT cells preferentially home to the sinusoidal areas of the splenic red pulp whereas both the αβ T cells ($TCR2^+$ and $TCR3^+$) are located in the peri-arteriolar sheaths (Cooper *et al*., 1991; Chapter 2).

In chicks, the intestinal epithelium harbours a large number of both αβ and γδ T cells. Among the intraepithelial lymphocytes (IEL), 60% of the γδT cells and 30% of the αβT cells express CD8αα homodimers. Cell transfer experiments using congenic chicks have shown that EID 14 or adult thymocytes do not contain any detectable CD8αα T cells. However, when TCRαβ or TCRγδ thymocytes were injected into congenic animals, they migrated to the gut and developed into CD8αα IEL. Analysis of the TCR $V\beta_1$ repertoire of CD8αα TCR $V\beta_1$ IEL has indicated that these cells migrate from the thymus at an early stage in the developmental process. Chicken TCRαβ $V\beta_2$ T cells are extremely rare in the intestine (Dunon *et al*., 1993; Imhof *et al*., 2000).

THE BURSA OF FABRICIUS, B CELL ONTOGENY AND IMMUNOGLOBULINS

Bursal and B Cell Development

The bursa of Fabricius is unique to birds and essential for the amplification and differentiation of B lymphoid progenitors. As described in Chapter 1, it has made an important contribution to discovering the dichotomy of the immune system into a thymus-dependent and humoral (bursal-dependent) arms (Glick *et al*., 1956; Cooper *et al*., 1965). The bursa of Fabricius develops in a similar way to the thymus, from an endo-mesodermal rudiment which is colonized by HC of extrinsic origin. In the chick, the bursal anlage appears on EID 4 as an epithelial bud emerging in the proctodeal region of the cloaca. This organ reaches its maximum size about 2 or 3 months after hatching. In contrast to the thymus, bursal invasion occurs during a unique, continuous period lasting only a few days between EID 8 and 14 (Houssaint *et al*., 1976). Typically, B cell antibody diversification occurs in the bursal follicles (see Chapter 4). At hatching a bursa contains approximately 10 000 follicles composed of an outer cortex of endodermal origin and a medulla of mesodermal origin. Each follicle contains about 100 000–150 000 B lymphocytes (Plate 3.4). Follicles do not form in a synchronous manner and new buds can appear between EID 11 and 14. Surgical removal of the bursa during the early embryonic period results in impairment of the humoral immune system. Bursectomy of late-stage embryos or neonate chicks causes a marked reduction in the number of circulating B lymphocytes and lack of ability to produce specific antibodies in response to antigenic challenge. This demonstrates that the

bursa provides a unique microenvironment essential for the proliferation and differentiation of B cells (Ratcliffe, 2006). The first surface IgM-positive cells are detected from EID 12 and at hatching more than 90% of bursal cells are mature B cells. During embryonic life, the bursa of Fabricius is productively colonized by a limited number of B cell precursors that have already undergone Ig gene rearrangement at sites of haematopoiesis, namely the para-aortic foci (see earlier) and the bone marrow. These Ig gene rearrangements occur in the absence of TdT, generating only minimal antibody diversity (Ratcliffe and Jacobsen, 1994). Cells which fail to express surface Ig are eliminated by apoptosis; only B cell precursors that productively rearrange the Ig gene express cell surface Ig and are selected for subsequent expansion in bursal follicles. Subsequently, the rearranged variable region, which generates Ig specificity, undergoes somatic diversification by a process of intrachromosomal gene conversion in which blocks of nucleotide sequence are transferred from the pseudo-V genes into the unique functional rearranged V_H and V_L genes (Plate 3.4(a)). This diversification mechanism by segmental gene conversion can result in the creation of an Ig repertoire of at least 10^{11} distinct antibody molecules (McCormack *et al*., 1991). A detailed description of the process can be found in Chapter 4.

Immunoglobulins

Three main classes of antibodies have been described for birds: IgM, IgA and IgY, the latter being the avian homologue of mammalian IgG (see Chapter 6). Immunofluorescence staining using antibodies against the different chicken Ig-μ and light (L) chains detected IgM synthesis by bursal cells as soon as recognizable lymphocytes appeared in the bursa, i.e. about EID 14 (Leslie and Clem, 1969). IgY-producing lymphocytes appeared in bursal follicles later, at around the time of hatching (equivalent to EID 21). Cells containing IgM were observed outside of the bursa from EID 17, whereas cells containing IgY chains were not detected at extra-bursal sites (spleen, caecal tonsils and thymus) until 4 days after hatching (Kincade and Cooper, 1971). Rapid expansion of IgM- and IgY-containing cells begins in the spleen on the 3rd and 8th days after hatching, respectively. Experiments using bursectomized chicks treated with antibodies against Ig-α and Ig-μ chains indicated that during ontogeny, IgM-forming cells are the direct precursors for IgY- and IgA-forming cells by a genetic switchover mechanism (Kincade and Cooper, 1971; Martin and Leslie, 1974).

The architecture of the chicken heavy (H) and light (L) chain loci is unique, in that only single functional V and J segments are present in both loci. Upstream of the functional V gene segment 80–100 pseudo-V_H genes are present in the IgH locus and 26 pseudo-V_L in the IgL locus (Ratcliffe and Jacobsen, 1994). About 16 D_H segments are present in the IgH locus (Reynaud *et al*., 1991; Plate 3.4(a)). The Ig chain gene rearrangements are not restricted to the bursa of Fabricius. The site for earliest detection of D_HJ_H rearrangement is the yolk sac, between EID 5 and 6. D_HJ_H rearrangements are first detected in the spleen at EID 6 or 7, in the blood at EID 8, in the bursa at EID 9 or 10, in the thymus at EID 9, in the bone marrow at EID 10 but in the para-aortic region only at EID 9 (Reynaud *et al*., 1992). A systematic lag for the other rearrangements (V_H to D_HJ_H, V_L to J_L) has been observed: EID 9 in the blood and the yolk sac, EID 8 or 9 in the spleen, EID 10 or 11 in the bursa, and EID 11 in the bone marrow and thymus. In the chicken, $V_HD_HJ_H$ and V_LJ_L rearrangements are observed simultaneously, indicating that there is no sequential rearrangement of IgH and IgL chains genes. This suggests in chicken, the lack of a pre-B cell stage containing a functionally rearranged $V_HD_HJ_H$ sequence prior to IgL gene rearrangement.

LYMPHOCYTE-DIFFERENTIATING HORMONES

Several studies have demonstrated the role of polypeptide hormones in T and B cell differentiation. Thymus extracts have been shown to induce selective T cell differentiation of precursor cells from the bone marrow *in vitro*. The active extract has been identified as a thymic hormone designated thymopoietin and shown to be synthesized by non-lymphoid, epithelial and dendritic cells (Goldstein *et al*., 1977).

Bursal extracts have also been shown to induce both B and T cell differentiation, however the effect on chicken B cells is dominant because, as the extract is diluted, the effects on B cell differentiation remain after the effects on T cells are lost (Brand *et al*., 1976). The B cell differentiating hormone isolated from the bursa of Fabricius has been called bursin. It has a tripeptide structure (lysyl–histidyl–glycyl-amide) and induces phenotypic differentiation of B cell precursors (Audhya *et al*., 1986). Using a B cell line, bursin was shown to increase the cyclic adenosine and guanosine monophosphates. Immunohistochemical staining with an antibody specific for bursin showed that expression is essentially restricted to follicular and dendritic reticular epithelial cells and to the basal layer of interfollicular epithelium in the mature bursa. If antibodies against bursin are inoculated intravenously at EID 13 they suppress the appearance of bursal IgM cells at EID17 suggesting that bursin may act on the emergence of IgM^+ cells early in bursal development (Otsubo *et al*., 2001). A role for activin A and B in B cell development has also been suggested. Expression of the two activin subunits increases from EID 18 until 1 week after hatching. Activin A is predominantly localized in the medullary epithelia whereas activin B is found in the follicle-associated epithelium suggesting that the two subunits have distinct roles in modulating the bursal microenvironment during B lymphocyte differentiation (Blauer and Tuohimaa, 1995).

Neurotrophin receptor-like proteins also have been identified in both avian primary lymphoid organs. Using immunohistochemical techniques, specific immunoreactivity has been detected for three of these tyrosine kinase receptors: TrkA-, B- and C-like. In the thymus, TrkA-like receptors are present in the medullary epithelial cells and in a subpopulation of cortical epithelial cells; TrkB-like receptors are present in the medullary dendritic cells and cortical macrophages and TrkC-like are found in the cortical and medullary epithelial cells. In the bursa, TrkA- and C-like receptors are found exclusively in the epithelial cells associated with follicle and the interfollicular epithelia. TrkC-like receptors are found in some medullary reticular epithelial cells. The selective localization of these receptors is consistent with the differential role of neurotrophin in the microenvironment of the primary lymphoid organs and, indirectly, with its effects on B and T lymphocyte differentiation (Ciriaco *et al*., 1996).

Glucocorticoid hormones have an important role in mammalian T cell selection. The recent identification of enzymes and cofactors required for glucocorticoid production, in both the bursa and thymus, suggests that these hormones could have an important role in development and selection of avian B and T lymphocyte. Moreover, the observation that glucocorticoid production occurs in the organ responsible for B development is a further step to elucidating the, as yet poorly defined, mechanism of B cell selection in birds (Lechner *et al*., 2001).

DEVELOPMENT OF THE IMMUNE RESPONSES

Early Immune Responses

Developing avian embryos and neonatal chicks are transiently protected against bacterial toxins, bacteria, parasites and viruses by maternal Ig transferred via the yolk (see Chapter 6). Maternal antibody can persist for up to a month after hatching (Hamal *et al*., 2006). The chick begins to develop its own defence mechanisms during embryonic life, but immunocompetence only appears a few days after hatching (Mast and Goddeeris, 1999). From the immunological point of view, the post-hatching period is crucial, since the chick is abruptly exposed to a wide range of environmental antigens and is not supplemented with further maternal immunity, such as that provided by mammalian colostrum. Immunization 1 day after hatching does not activate antibody production, probably due to incomplete structural organization of the secondary lymphoid tissues in neonate. However, 1 week later, immunization with the thymus-dependent antigen bovine serum albumin generated an effective humoral response with specific antibody production (Mast and Goddeeris, 1999).

Antibody Isotype Switching and Hypersensitivity Reaction

As in mammals, after antigenic challenges the initial synthesis of specific IgM is followed by production of increasing amounts of lower-molecule-weight antibodies (IgY). Isotype switching during the course of an immune response occurs in the germinal centres where B cell memory develops (Yasuda *et al*., 2003). The germinal centres are also the site for hypermutation, a process necessary for the affinity maturation of the antibodies involved in the secondary immune response (Arakawa *et al*., 1996). Immediate hypersensitivity (IH) and delayed hypersensitivity (DH) reactions have been described for chickens following immunization with soluble antigenic proteins, and these reactions seem to be age dependent (Zhu *et al*., 1999). Indeed when injected with human γ-globulin, 6- to 12-week-old chickens exhibited a significantly greater DH reaction than 3-week-old birds. Consistent with this, IH can be lethal in adults but appears less severe in young chicks. IH reactions can be blocked by anti-histamines and are probably induced by degranulation of mast cells (Parmentier *et al*., 1993). In chickens, gut-associated lymphoid tissue, which provides an important enteric protection in the absence of oral maternal antibodies, is functionally mature as early as 4 days after hatching but the secretory IgA response against enteric antigens develops only gradually, maturing towards the end of the second week of age (Bar-Shira *et al*., 2003).

Allograft Rejection

Birds show acute rejection of allografted tissue, with a rapid primary response and clear-cut secondary response with memory. Chicks become fully competent in their alloimmune responses within a few days of hatching. Acute rejection is controlled by the MHC (Schierman and Nordskog, 1961). Skin grafts between incompatible chickens show the onset of rejection at about 7 days after the first graft but only 3–4 days after the second graft in a series. Graft-versus-host (GVH) reactions were first discovered using the avian system, as described in Chapter 1. GVH responses occur when cells from an immunologically competent bird are implanted in an immunologically immature embryo or an immunosuppressed individual; the alloimmune reaction of the lymphoid cells of the donor against the host is detectable about 4 days after the cells are inoculated. In the chick embryo this is manifest by lesions on the chorio-allantoic membrane and by the enlargement of the host's spleen (splenomegaly). Blood cells from a late embryo (20-day-old embryo) are capable of causing GVH reactions when inoculated into a young embryo. The host chick becomes able to resist to GVH within a few days of hatching once it has become fully immunocompetent (Watabe and Glick, 1983; Desveaux-Chabrol *et al*., 1989).

CONCLUSIONS

Aves are a highly evolved and successful class of vertebrates and share a number of common features with that other highly-evolved class, the mammals. Some of these characteristics are inherited from a common ancestor whilst others are due to convergent evolution. Birds have a number of specialized adaptations, one being the development of the bursa of Fabricius as a primary lymphoid organ. Although functional delineation of the B and T cell lineages and specialized microenvironments for their differentiation occur in other vertebrate classes, it is only in bird that the site for primary differentiation of B lineage cells is restricted to an anatomically distinct organ. This clear-cut separation of B and T cell lineages provides an extremely useful model, for immunological studies. For example, a "T cell individual" in which the B cell population is depleted (bursectomy) while T cell system remains intact can be produced by a number of different means. Hence, studies on the diversification of antibody repertoires using the chicken has revealed some interesting and unusual features – such as somatic gene conversion – which achieves an impressive immunoglobulin repertoire, the equal of that in mammals. Bursectomy is relatively straightforward and has been employed successfully to investigate the relative contributions of antibodies and cell-mediated immune responses in immune protection.

Unfortunately creating an experimental "B cell individual" by ablating T cells using thymectomy is a much more difficult proposition, and has been achieved on relatively few occasions (Cihak *et al*., 1991; Dunon *et al*., 1993). With ease of access to large numbers of inbred lines and precisely-timed stages in embryonic development, birds provide a tremendously valuable model for studying the mechanisms involved in immunological development as well as contribution of the different responses in immune resistance.

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4

B CELLS, THE BURSA OF FABRICIUS AND THE GENERATION OF ANTIBODY REPERTOIRES

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INTRODUCTION

The bursa of Fabricius is critical for normal development of the B lymphocytes responsible for antibody production. The bursa is unique to birds and was first implicated as having a role in the development, or generation, of antibody responses about 50 years ago with the discovery that surgical removal of the bursa from neonatal chicks impaired subsequent antibody responses to *Salmonella typhimurium* type O antigen (Glick *et al*., 1956). Since that time, it has become clear that the bursa is the primary site of B cell lymphopoiesis in birds, and that avian B cell lymphopoiesis has characteristics distinguishing it from pathways of B cell development in human or rodent models (Ratcliffe, 1989, 2006; Sayegh *et al*., 2000; Pike and Ratcliffe, 2002; Pike *et al*., 2004a). In this chapter, I will discuss the development of B lymphocytes and the function of the bursa in B cell development.

The bursa itself is colonized during embryonic development by lymphoid precursors that expand and mature in the bursa before emigrating to the periphery where they have the potential to take part in immune responses. As a consequence, we can consider B cell development as occurring in three distinct stages, pre-bursal, bursal and post-bursal and, as we will see, each of these stages plays a fundamentally different role in B cell development.

Prior to discussing the development of B cells at the cellular level, it is important to consider the function of the B lymphocyte lineage, which is to generate antibodies following exposure to foreign pathogens. As a consequence, B cell development also requires the generation of a repertoire of antigen-specific B cells that can provide such protective antibodies. The specificity of B cells is a function of the immunoglobulin (Ig) genes that encode antibody molecules and so, in the first part of this chapter, I will discuss the means by which Ig genes in birds generate a diverse repertoire of B cell specificities.

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GENERATION OF AVIAN ANTIBODY REPERTOIRES

In rodents and primates antibody diversity is generated by the random assortment of genetic elements that encode the variable region domains of Ig heavy and light chains in a process called Ig gene rearrangement (Tonegawa, 1981). It has become clear over the last 20 years that, although avian Ig genes in birds undergo gene rearrangement, antibody diversity is generated by a process of somatic gene conversion (reviewed in McCormack *et al.*, 1991). This process is fundamentally different from the process of Ig gene rearrangement and, based on the number of different variable regions that can be generated, appears to be at least as efficient as gene rearrangement as a mechanism for generating antibody diversity.

Ig Light Chain Rearrangement

Chickens contain only one Ig light chain, in contrast to rodents and primates that contain both κ and λ light chain loci. It is unclear whether this represents an evolutionary branching of birds prior to the duplication of light chain loci seen in mammals, or whether one of two light loci was not maintained in birds. Nonetheless, chickens in particular and, as far as we know, birds in general contain the products of only one functional Ig light chain locus. This locus is more closely related to the λ light chain locus than to κ; a relationship based not just on protein or DNA sequence homology but on the structure and organization of the recombination signal sequences (RSS) required for the generation of functional Ig light chains.

The light chain variable (V_L) protein domain is encoded by the rearrangement of a V_L gene with a J_L segment. In chickens there is only one functional V_L gene and only one J_L segment and as a consequence all chicken B cells undergo essentially the same gene rearrangement event (Reynaud *et al.*, 1985, 1987; Thompson and Neiman, 1987). This is in striking contrast to V_L–J_L rearrangement in rodents and primates where large families of V_L genes can undergo rearrangement to one of several distinct J_L segments. At a functional level, the consequence of this is that while rodents and primates can generate substantial amounts of VJ_L diversity by the process of rearrangement itself, in chickens the process of Ig gene rearrangement at the light chain locus generates minimal VJ_L diversity (Fig. 4.1).

The mechanism of Ig gene rearrangement appears to be highly conserved between birds and mammals. The products of RAG-1 and RAG-2 genes generate a recombinase complex that is responsible for recognizing the RSS directly flanking gene segments that undergo recombination. RSS comprise a 7 nucleotide conserved heptamer sequence that is typically palindromic and a conserved 9 nucleotide AT-rich nonamer. The conserved heptamer and nonamer are separated one from another by so-called spacer sequences that can be either 12 or 23 base pairs long. As a general rule, the sequence of the spacer is much less important than the length of the spacer suggesting that the relative orientation of the heptamer and nonamer are important for the recombination process. In this regard, the 12 and 23 base pair spacer lengths approximately correspond to one and two turns of the DNA helix, respectively. Rearrangement takes place between a sequence flanked by an RSS with a 12 base pair spacer (RSS_{12}) and a sequence flanked by an RSS with a 23 base pair spacer (RSS_{23}). Thus at the chicken light chain locus the unique functional V_L1 sequence is flanked by an RSS_{23} and the unique J_L segment is flanked by an RSS_{12}. In chicken B cell precursors, the co-expression of RAG-1 and RAG-2 therefore coincides with cells undergoing rearrangement of Ig genes (Reynaud *et al.*, 1992).

At a molecular level, V_L–J_L rearrangement in chickens appears indistinguishable from V(D)J recombination seen in other species. RAG-1 and RAG-2 together cause a single-stranded nick at the junction between the RSS and the coding sequence. The nicked end displaces the other strand in a transesterification reaction that results in the coding sequence forming a hairpin structure and leaves the RSS as blunt open-ended DNA. Opening of the hairpin structure frequently occurs asymmetrically resulting in deletion of coding nucleotides or the addition of palindromic (P) nucleotides to the VJ_L junction. In contrast to rodent or human VJ_L junctions where the enzyme terminal deoxyribonucleotidyl transferase (TdT) adds random non-templated (N) nucleotides to

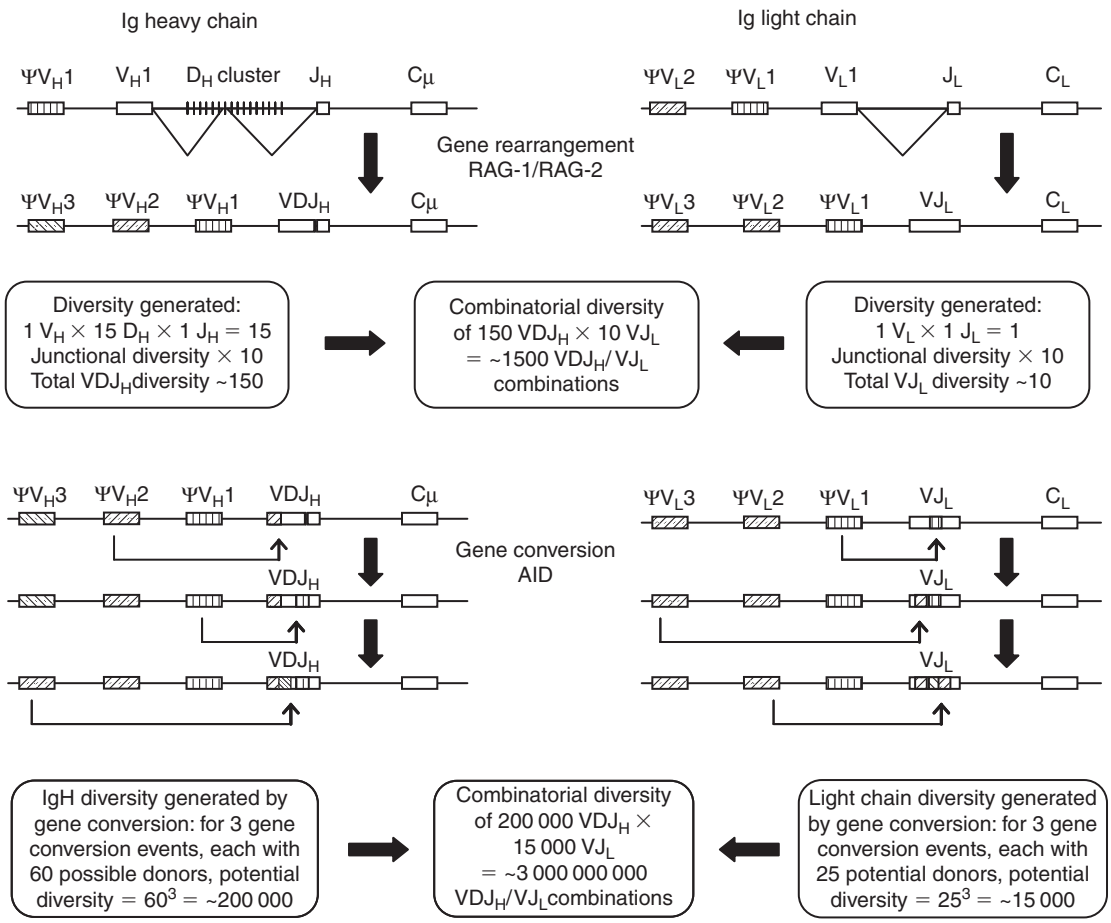

FIGURE 4.1 The generation of antibody diversity in the chicken. Chicken antibody diversity is generated by gene rearrangement followed by gene conversion. At each stage of the process an estimate is given of the amount of potential diversity among the resulting VDJ_H and VJ_L genes and in the B cell population overall. Estimates of diversity due to rearrangement may be high due to sequence redundancy among the D_H cluster. Estimates of diversity as a consequence of gene conversion may be low since individual gene conversion events may affect variable numbers of amino acids and somatic point mutations arising in the V regions have not been taken into consideration.

coding ends prior to their joining, there is no evidence for the presence of N nucleotide additions in chicken VJ_L junctions. Indeed it was the presence of P nucleotides in the absence of N nucleotides that provided crucial insights into the definition of the hairpin structure intermediate in V(D)J recombination (McCormack *et al*., 1989a). The absence of N nucleotides appears to be the only mechanistic difference between V(D)J recombination in chickens and mammals. As far as is known homologous enzymes are responsible for cleavage, orientation of the recombinationally active ends and ligation of the VJ_L junction in mammals and birds.

While chickens are clearly the best studied avian species, an analysis of Ig light chain rearrangement in the Muscovy duck has revealed that there are four functional V_L genes, each of which can undergo rearrangement to a unique J_L (Pandey *et al*., 1993). As a consequence, it is likely that different species of birds may use a different numbers of light chain V_L genes and chickens simply represent the extreme situation where the entire light chain repertoire is derived from a single V_L–J_L combination. Nonetheless, while some birds may use more V_L genes than others, it is likely that in all avian species the process of somatic gene conversion is the dominant mechanism for generating antibody repertoires.

Ig Heavy Chain Rearrangement

As with the chicken light chain, gene rearrangement contributes little to the generation of chicken Ig heavy chain diversity. In common with other species, the Ig heavy chain V_H protein domain is encoded by three gene segments, variable (V_H), diversity (D_H) and joining (J_H). However, in contrast to other, especially mammalian, species where there are large families of V_H, D_H and J_H segments, the chicken V_H domain is generated by recombination of unique V_H and J_H gene segments with one of a family of D_H elements (Reynaud *et al.*, 1989). Although there are approximately 15 D_H segments in the chicken, these segments show much lower levels of diversity than equivalent segments from mouse or human; indeed many of the chicken D_H elements are identical to one another at the protein level (Reynaud *et al.*, 1991).

The chicken V_H, D_H and J_H sequences are also flanked by the appropriate RSS to guide the recombination process. At a molecular level, chicken heavy chain rearrangement occurs in the same way as light chain rearrangement, as is the case in rodents and primates. Similar to rearrangement at the chicken Ig light chain locus, there appears to be no N nucleotide additions induced as a consequence of TdT expression, but significant nucleotide deletion and/or P nucleotide addition (Reynaud *et al.*, 1989, 1991).

The assembly of the chicken heavy chain VDJ_H exon is ordered, as it is in mammals, with D_H to J_H rearrangement preceding the rearrangement of the unique V_H1 gene into the preformed DJ_H complex. Nonetheless, in contrast to mammalian VDJ_H rearrangement, in the chicken there is a high frequency of D_H to DJ_H rearrangement. Thus during embryonic development, many examples of DDJ_H, and even $DDDJ_H$ rearrangements have been isolated from the chicken bursa. Such DDJ_H and $DDDJ_H$ rearrangements retain their capacity to rearrange to the unique V_H1 gene and so potentially could contribute to the diversity generated by recombination. Nonetheless, at this stage the biological importance of such $VDDJ_H$ or $VDDDJ_H$ sequences is unclear (Reynaud *et al.*, 1989, 1991).

The generation of DDJ_H sequences requires D_H to D_H rearrangement and since chicken D_H elements are flanked on both sides by RSS_{12} sequences, such rearrangement should be in contravention of the 12/23 rule of recombination. Recent evidence, however, suggests that such 12/12 recombination is indeed possible at a significant frequency due to the sequence of the 12 base pair spacers typically found in chicken D_H RSS. This provides evidence that the sequence of the spacers in RSS may be more important in regulating the efficiency of V(D)J recombination that was first appreciated (Agard *et al.*, 2007).

Due to the molecular mechanism of the recombination process, rearrangement is characteristically inefficient in the sense that two-thirds of all rearrangements will generate out of frame products. This is also true in the chicken despite the lack of N nucleotide insertions at V(D)J junctions. In turn, this means that only one-third of light chain gene rearrangements will generate a functional light chain product. At the chicken heavy chain locus the D_H element has to be used in a specific reading frame (as discussed in Pike and Ratcliffe, 2002) and so only one in nine VDJ_H sequences generated will maintain the correct reading frame of both D_H and J_H relative to V_H.

GENERATION OF Ig MOLECULES BY V(D)J RECOMBINATION

In mammals, VDJ_H rearrangement typically occurs prior to, and is required for, VJ_L rearrangement. In chickens, however, it has become clear that following initial DJ_H rearrangement, the rearrangement of V_H1 to DJ_H (or DDJ_H) and V_L1 to J_L occur stochastically during the same window of time. Thus while cells have been isolated by retroviral transformation that contain VDJ_H rearrangements in the absence of VJ_L rearrangements, other retrovirally transformed cells contained VJ_L rearrangements in the absence of VDJ_H rearrangements (Benatar *et al.*, 1992). This is consistent with the pattern of rearrangements seen in the developing embryo (Reynaud *et al.*, 1992) and means that while the molecular mechanism of rearrangement is indistinguishable between mammals and birds, the way in which recombination is regulated is clearly different. Why this

difference has evolved remains unclear. However, since the process of gene rearrangement in mammals is the mechanism by which the primary repertoire is generated, amplification by cellular proliferation of successful heavy chain rearrangement prior to light chain rearrangement would increase the potential for generating maximal diversity. In contrast, since gene rearrangement in the chicken generates minimal diversity, amplification of productive heavy chain rearrangement prior to light chain rearrangement might increase the number of cells containing rearrangements, but would not increase the diversity of the repertoire contained within this population.

The coincident rearrangement of chicken V_H and V_L genes represents an important difference between avian and mammalian B cell development. In mammals, productive heavy chain VDJ_H rearrangement leads to the generation of full length μ heavy chains that associate with the surrogate light chain molecules VpreB and $\lambda 5$. Together VpreB and $\lambda 5$ associate with the μ heavy chain to form the multimeric pre-B receptor $(\mu/\text{VpreB}/\lambda 5)_2$. Expression of this receptor complex inhibits further heavy chain rearrangement and is required to support the progression of mammalian B cells to the point where they begin to rearrange at the light chain locus (reviewed in Melchers, 2005). In chickens there is no evidence to support the existence of a homologous complex to the mammalian pre-B cell receptor (BCR) and chicken homologues to VpreB and $\lambda 5$ have not been identified, despite the availability of extensive chicken genome sequence data. Since the expression of the pre-B receptor defines the pre-B cell in the mammalian bone marrow or foetal liver, it has become clear that chickens and, likely by extension, other birds do not contain pre-B cells.

The functionally rearranged chicken light chain includes a leader sequence that encodes the leader peptide required for translocation of the nascent polypeptide chain into the endoplasmic reticulum, the VJ_L sequence that encodes the V_L protein domain and the unique C_L exon that encodes the C_L protein domain. The post-transcriptional and post-translational processing required to generate the mature light chain that contains the V_L and C_L domains appear to be indistinguishable from that seen in mammals.

Similarly, the functionally rearranged heavy chain includes the leader sequence, the VDJ_H sequence that encodes the V_H protein domain and the Cμ exons that encode the constant region of the IgM heavy chain. As in other species, the Cμ constant region gene is found immediately downstream of the VDJ_H region and IgM is the first Ig molecule expressed by developing B cells. Downstream of the Cμ gene are the constant region genes for IgG and IgA, as will be discussed in more detail below.

GENERATION OF Ig DIVERSITY BY SOMATIC GENE CONVERSION

While chicken Ig gene rearrangement generates minimal V region diversity, the diversity of antibodies produced in the chicken appears to be no less than in mammals. The resolution of this paradox came from the characterization of the chicken Ig light chain locus. Upstream of the functional V_L1 gene there is a family of sequences which are highly homologous to the V_L1 sequence; these show a number of key differences that render them non-functional. As such, these genes have been described as pseudo-V_L genes (ΨV_L) although it has become clear that these ΨV_L genes do indeed contribute to the development of avian antibody repertoires (Reynaud *et al*., 1987; Thompson and Neiman, 1987). Nonetheless, the ΨV_L genes lack 5′ promoter and leader sequences and also lack functional RSS. This means that the ΨV_L sequences cannot themselves undergo recombination to the J_L segment. In addition, many of the ΨV_L sequences contain 5′ and/or 3′ truncations as compared to the functional V_L1 gene.

At the light chain locus there are approximately 25 ΨV_L genes and sequence comparisons show that the major blocks of diversity among these ΨV_L genes occur at positions that correspond to the hypervariable or complementarity determining regions (CDR) of the Ig light chain V region gene, corresponding to the antigen binding site of the Ig molecule. Thus the diversity of the Ig repertoire is maintained at the germline level among the family of ΨV_L genes rather than among the functional V gene families as is the case in mammals.

The result of an individual gene conversion event at the chicken Ig light chain locus is that a stretch of sequence in the rearranged V_L1 gene is replaced by homologous sequence derived from one of the upstream pseudogenes. This is a more variable process than simply a replacement of one V gene with another, since the length of the exchanged sequence can vary extensively from one gene conversion event to another. Gene conversion events have been identified that span anywhere from a minimum of about 10 nucleotides up to more than 300 nucleotides, covering most of the V_L sequence. Thus even under circumstances where we consider single gene conversion events involving a particular rearranged V_L sequence and a specific donor ΨV_L gene, gene conversion can generate multiple different sequences, which translates into light chain repertoire diversity.

At the light chain locus, the 25 ΨV_L genes are distributed in about 20 kb immediately upstream of the functional V_L1 gene. This spacing is far more compressed than is evident in rodent or primate V gene clusters, where V genes are typically spaced about 10 kb from one another. It is widely regarded that the reason for the wide spacing of V genes in rodents and primates is to minimize the activation of upstream V gene(s) following VJ_L rearrangement. Closer spacing would put the promoters of upstream V regions, i.e. V genes that have not themselves undergone rearrangement, within range of enhancer sequences in the J–C intron leading to transcript production. Since the chicken ΨV_L genes do not themselves include promoters, close spacing would not result in ΨV_L transcription. In addition to being closely spaced, many of the ΨV_L genes are arranged in a head-to-head, or tail-to-tail, orientation, placing many of the ΨV_L genes in the opposing orientation as compared to the V_L1 gene.

Analysis of the usage of different pseudogenes as sequence donors in gene conversion events has revealed a number of properties that underlie the process of gene conversion. These analyses have been based on defining ΨV_L usage in B cells typically from the embryo or neonate that contain relatively few gene conversions making it easier to define individual conversion events (McCormack and Thompson, 1990).

The first general rule is that ΨV_L genes closest to the functional V_L1 gene are used more frequently as donor genes. This has been reasonably interpreted as indicating a requirement for alignment of donor and recipient sequences during gene conversion. It has also become clear that those sequences showing greatest homology to the recipient sequence are used as sequence donors more frequently than others. Again this is consistent with a requirement for sequence alignment during gene conversion. Finally, those ΨV_L genes that are in the opposing orientation to the V_L1 gene tend to be used as sequence donors more frequently than those in the same orientation. This has been interpreted as evidence that alignment between the ΨV_L and functional gene can frequently occur by folding back of the DNA strand to align the donor and recipient sequences. This is a reasonable interpretation since gene conversion has been shown to occur in cis orientation, i.e. the donor and recipient sequences must be on the same DNA strand, and that ΨV_L sequences on the allelic locus do not contribute to gene conversion events (Carlson *et al*., 1990).

Although gene conversion at the heavy chain locus is not as clearly defined as it is at the light chain locus, it is clear that the extensive heavy chain repertoire generation occurs by gene conversion (Reynaud *et al*., 1989, 1991). Again donor sequences are derived from upstream pseudogenes, although in the case of heavy chain gene conversion, the donor pseudogenes include both V_H and D_H homologous sequences. As a consequence, the junctional diversity generated by rearrangement at the VD_H junction is frequently overwritten by gene conversion events (Reynaud *et al*., 1991).

A major breakthrough in understanding the molecular mechanism of gene conversion came with the identification of the enzyme activation-induced cytidine deaminase (AID) as being essential for gene conversion (Arakawa *et al*., 2002). AID was initially cloned from murine germinal centre B cells and targeted deletion of AID in mice resulted in a complete absence of somatic hypermutation and class switch recombination (Muramatsu *et al*., 2000). A subset of human immunodeficiencies that are characterized by an absence of class switching and point mutations were also determined to be a consequence of deficiencies in functional AID expression. Targeted

deletion of AID in a chicken B cell line, DT40, which normally continues to undergo gene conversion *in vitro*, also resulted in a complete inhibition of gene conversion events and it has now become clear that AID is indispensable for gene conversion in chicken B cells.

AID is homologous to APOBEC1, an enzyme responsible for editing RNA by methylating cytosine residues to form uracil resulting in changes in translated protein sequence. While it was initially unclear whether AID initiated V gene mutation by targeting the Ig V region directly or by modifying RNA encoding a putative "mutator" protein, there is now strong evidence accumulating that AID can indeed directly modify single-stranded DNA in the V gene itself (Bransteitter *et al*., 2003; Dickerson *et al*., 2003). Methylation of C residues to form U results in the modification of a dC–dG base pair to a dC–dU base pair mismatch which is then subject to error repair. It is likely the nature of the error repair process invoked that defines the nature of the resulting modification in the V gene, gene conversion, somatic hypermutation or class switch recombination (Martin and Scharff, 2002).

Since AID targets single-stranded DNA, the question arises as to how such single-stranded DNA is generated at the Ig locus and why AID-induced mutations are preferentially targeted to the Ig loci. One possibility, that has been widely considered, is that as transcription of the functional Ig V gene proceeds, the transcription bubble causes unwinding of the DNA and strand separation to allow the formation of the nascent RNA chain. The non-transcribed DNA strand is therefore single stranded and could therefore act as a substrate for AID-induced methylation of C residues. This model could clearly explain why the target for gene conversion is the rearranged V region gene since, although the ΨV_L genes show considerable homology to the functional gene, they are not transcribed due to their lack of promoters. Similarly, in B cells containing one rearranged V_L1 gene with the other V_L1 gene in germline configuration, gene conversion is restricted to the rearranged allele (Thompson and Neiman, 1987). Nonetheless, since many genes are being transcribed at high levels in B cells that are undergoing gene conversion, the question remains as to how AID is preferentially targeted to the Ig loci.

While AID targets C residues in the V gene sequence, not all C residues are equally susceptible to AID-induced modification. It has long been appreciated that somatic hypermutation, also initiated by AID, is targeted to the C residue in hotspots defined by W(A/G)R(G/T)C motifs. At present it is unclear whether this represents preferential binding of AID to WRC motifs, as opposed to other C containing motifs, or whether initial AID binding is independent of sequence, with the specificity coming from a WRC preference for targeted methylation. Nonetheless, the overall consequence is preferential dC to dU mutations in WRC motifs. Comparison of the sequence of Ig V genes with C region sequences reveals that there are much higher frequencies of WRC motifs in the V gene sequences, typified by a frequent usage of AGC to encode serine. It would appear therefore that the V gene sequences have a codon usage that enriches for codons susceptible to AID-induced modification.

Following AID-catalyzed dC to dU modification, the resulting dU–dG base pair represents a mismatch in the DNA duplex. Such mismatches are typically repaired by eukaryotic cells to minimize the accumulation of mutations. There is now considerable evidence accumulating that the nature of the error repair processes invoked can determine the nature of the modification induced at the Ig locus. Thus XRCC2, XRCC3 and RAD51B, all of which are paralogues of the RAD51 gene in yeast, as well as RAD54 and uracil-DNA glycosylase all play a role in gene conversion as targeted deletion of these genes in the DT40 cell line inhibits gene conversion (Sale *et al*., 2001; Di Noia and Neuberger, 2004). In contrast, several of the molecules involved in gene rearrangement including RAG-2 (Takeda *et al*., 1992), Ku70, and DNAPKcs as well as the RAD52 gene product do not play a role in gene conversion.

While deletion of AID precludes both gene conversion and somatic hypermutation, deletion in DT40 cells of several of the molecules selectively involved in gene conversion, particularly XRCC2, XRCC3 or RAD51B, results in DT40 lines that undergo somatic hypermutation, again supporting the contention that gene conversion and somatic hypermutation represent distinct mechanisms by which a common AID-induced lesion is repaired.

Implications of Gene Conversion on Allelic Exclusion

In rodents and primates, a high proportion of B cells contain one functionally rearranged heavy chain and one heavy chain allele in a non-productively rearranged conformation. The same situation is true at the light chain locus. The non-productively rearranged alleles never participate in the repertoire of B cell specificities since the somatic hypermutation mechanisms that diversify rodent and primate V regions following their rearrangement do not result in changes in the length of the V(D)J junctions and so cannot restore a productive reading frame to an out-of-frame rearrangement.

Gene conversion, on the other hand, results in the replacement of V gene-encoded sequence with sequences derived from upstream ΨV genes and it is clear that such gene conversion events are not simply a base for base substitution, since there are many examples of gene conversion changing the length of the resulting V_L sequence. This is due to the presence of different number of codons, typically in the hypervariable regions of different pseudogenes. As a consequence, it is reasonable to suggest that gene conversion might have the potential to "overwrite" a non-productive gene rearrangement with a gene conversion event spanning the VJ_L junction that could restore the correct reading frame for a productive VJ_L sequence. The net result would be the potential for individual B cells that initially contain one productively rearranged allele and one non-productively rearranged allele to undergo gene conversions that would result in both alleles being productively expressed (Sayegh *et al*., 1999b). This in turn would lead to the generation of individual B cells with more than one specificity, a situation that the developing immune system appears to take great pains to prevent.

Chicken B cells, however, contain one VJ_L rearrangement with the other allele remaining in germline configuration. Similarly at the heavy chain locus, each chicken B cell contains one heavy chain VDJ_H rearrangement with the other allele, typically either containing a DJ_H rearrangement or remaining in germline configuration. As a consequence, gene conversion cannot convert a non-productive rearrangement to one that is productive and so the product of a single allele is expressed by the B cell. The expression of a single allele is known as allelic exclusion and the allelically excluded nature of Ig gene expression is therefore maintained in birds at the level of gene rearrangement, whereas in rodents and primates it is maintained at the level of functional protein expression.

DEVELOPMENT OF AVIAN B CELLS

Pre-bursal B Cell Development

The development of avian haematopoietic lineages has been discussed in detail in Chapter 3 but there are aspects of early B cell development that should be re-emphasized at this time. Early studies based on grafting bursal rudiments between chicken and quail embryos clearly demonstrated that the B cell progenitors responsible for colonizing the bursa and forming the B cell lineage are derived from extra-bursal tissue and colonize the bursa from about the 8th to the 14th egg incubation day (EID; Le Douarin *et al*., 1975; Houssaint *et al*., 1976).

Cell transfer studies in which embryo spleen cells were transferred into immunocompromized recipient embryos demonstrated the presence of precursors with the potential to colonize both the thymus and the bursa. Importantly, these studies demonstrated that pre-bursal, but not pre-thymic cells, expressed the B cell surface antigen chB6 (sometimes referred to as Bu-1 antigen), demonstrating that the fate determination of the lymphocyte lineage to T cell and B cell potential occurs prior to the migration of pre-bursal cells to the bursa (Houssaint *et al*., 1991; Fig. 4.2). This presents an interesting counterpoint to the fate determination of murine lymphoid precursors, where strong evidence has been presented that activation of Notch 1 expressed on lymphoid precursors invokes a T cell fate, whereas the lack of Notch 1 activation leads to the default B cell fate (reviewed in Schmitt and Zuniga-Pflucker, 2006). The high-level expression of Notch 1 ligands in the mammalian thymus provides a model in which uncommitted precursors that

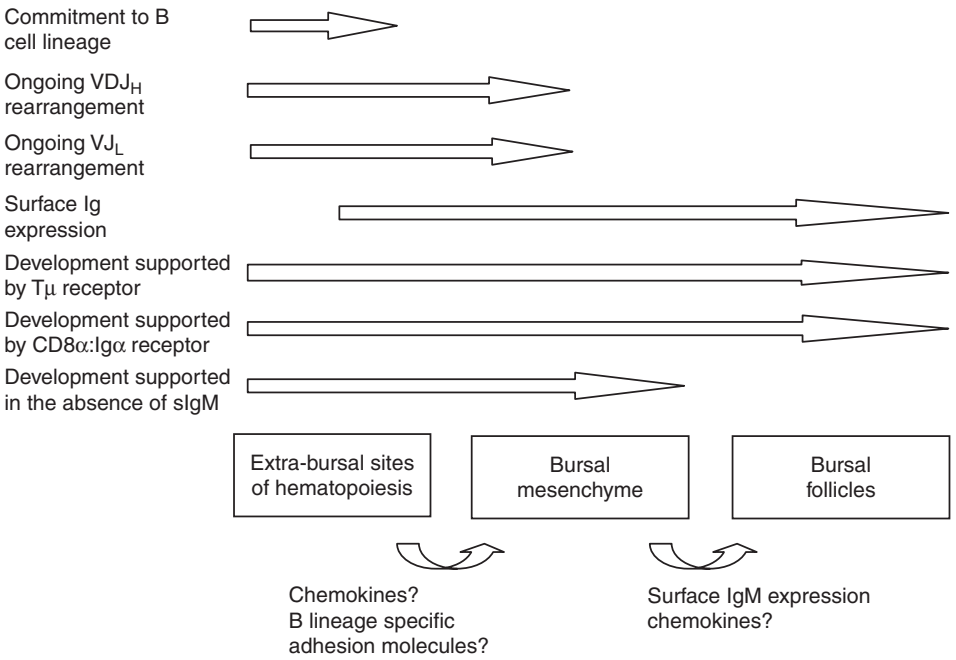

FIGURE 4.2 Requirements for the early development of chicken B cells. The early stages of B cell development are divided into pre-bursal, bursal mesenchyme and bursal follicle. The requirements for each stage of development are indicated by arrows. The text at the bottom suggests potential influences that govern the movement of cells from one compartment to the next.

seed the thymus are induced into the T cell lineage whereas precursors remaining in the bone marrow develop along the default B cell lineage (Schmitt and Zuniga-Pflucker, 2006). In the chicken, the same situation might occur, where precursors that colonize the thymus are induced to develop into T cells whereas precursors that do not colonize the thymus adopt an alternative fate(s). Since the default fate of lymphoid precursors in the absence of Notch 1 activation is the B lineage, it would suggest a restricted expression of appropriate Notch ligands in peripheral haematopoietic sites. Nonetheless, there is strong evidence that commitment to the B cell lineage occurs early in embryonic life, prior to the colonization of the embryo bursa.

The rearrangement of Ig genes is an essential stage in the development of the B cell lineage. Several lines of evidence have directly demonstrated that Ig gene rearrangement can occur before the B cell progenitors colonize the bursa. Thus, both Ig heavy and light chain rearrangements have been isolated from a variety of haematopoietic tissues and B lineage precursors with the capacity to colonize the bursa have been isolated from embryo bone marrow and shown to contain Ig rearrangements and express surface(s) Ig (Ratcliffe *et al*., 1986; Mansikka *et al*., 1990; Benatar *et al*., 1991; Reynaud *et al*., 1992). It is now clear that the bursal microenvironment is not required for the induction of RAG-1/2 expression and the rearrangement of chicken Ig genes.

Colonization of the Bursa by B Cell Progenitors

The bursal epithelium develops as an outgrowth of the urodeal membrane early in embryonic life. It is colonized by B cell progenitors in a single wave during the middle week of embryonic development and the colonization of the bursal microenvironment by B cell progenitors represents a critical stage in the development of the B cell lineage. As discussed above, there is strong evidence that the lymphoid progenitors that colonize the bursa are already committed to the B cell lineage and can have already undergone the process of Ig gene rearrangement. Nonetheless, many questions remain about the mechanisms underlying the functional colonization of the

bursa. In particular, it is important to consider the functional colonization of the bursa as occurring in two discrete stages. The first stage represents the migration of B cell progenitors into the bursal mesenchyme and the second distinct stage represents the colonization of lymphoid follicles within the bursa.

Based on parabiosis experiments in which the blood circulation of two embryos is joined, it is clear that B cell progenitors can migrate to the bursa through the blood (Pink *et al*., 1985). This is further supported by the demonstration of pre-bursal cells containing Ig gene rearrangements in the blood (Benatar *et al*., 1991). Clearly, however, embryo blood contains many other lineages of cells and so the question remains as to how the bursal mesenchyme selects B cell progenitors as opposed to other cell types. While it is clearly possible that all lineages traffic through the bursal mesenchyme with only B cell progenitors being retained, this seems unlikely based on the relatively high concentration of B cell progenitors evident in embryo bursal sections. Rather more likely is the possibility that blood-borne B cell progenitors selectively transit across vascular epithelia in the bursa into the mesenchyme. This might be analogous to the selective transit of naïve lymphoid cells across high endothelial venules in the lymph node or the transit of leukocytes across inflamed epithelia at sites of infection.

This model would predict the expression of specific adhesion molecules on the vascular epithelia of the bursa and complementary adhesion molecules selectively expressed on blood-borne B cell progenitors. At this time such molecules have not been defined. Nonetheless, an interesting parallel may exist in the rabbit appendix which, like the avian bursa, is a tissue which becomes colonized by B cell progenitors that subsequently undergo repertoire diversification by somatic gene conversion (Knight, 1992). In the rabbit, B cells with the capacity to colonize the appendix express CD62L (L-selectin), a homing receptor that binds ligands selectively expressed on specific vascular epithelia. A ligand for CD62L, PNAd, is expressed on the luminal side of high endothelial venules in B cell areas of the appendix and blocking CD62L binding to PNAd reduced the entry of B cells into the appendix. Thus, colonization of the rabbit appendix likely involves interactions between CD62L and PNAd occurring on the vascular epithelium (Sinha *et al*., 2006). In other models, CD62L ligation typically induces a rolling behaviour on the epithelial surface promoting interactions with locally produced chemokines and stronger interactions that involve integrin binding, ultimately leading to extravasation. At this point, this type of model fits the available data on how the bursal mesenchyme is colonized by B cell progenitors and begs the question as to the identity of the specific molecules involved.

Ig gene rearrangement can occur extra-bursally demonstrating that the bursal microenvironment is not required for the induction of gene rearrangement. Moreover, Ig gene rearrangement leading to sIg expression can be completed outside the bursal microenvironment. Nonetheless, analysis of the status of gene rearrangements in chicken B cell progenitors isolated from the embryo bursa prior to about 15 EID has demonstrated the presence of a high frequency of non-productive rearrangements (Reynaud *et al*., 1987). Similarly, clones of B cell progenitors isolated by retroviral transformation from 14 EID bursae frequently contain partial gene rearrangements, i.e. DJ_H alone, DJ_H and VJ_L, or VDJ_H alone (Benatar *et al*., 1992). As a consequence, it is clear that while V(D)J recombination can occur reaching completion extra-bursally, the initial colonization of the bursal mesenchyme does not require sIg expression. It is therefore likely that those B cell progenitors that colonize the bursa prior to completing V(D)J recombination can continue to rearrange in the bursal mesenchyme, consistent with the expression of both RAG-1 and RAG-2 in the embryo bursa (Reynaud *et al*., 1992). Importantly therefore, the process of V(D)J recombination can be completely dissociated from the regulation of colonization of the bursal mesenchyme.

Under normal circumstances, the bursa is colonized by pre-bursal cells from about day 8 to 15 EID. Experimentally, the capacity of pre-bursal cells to home to the bursa can be demonstrated by the ability of embryo bone marrow cells to colonize the bursa of recipient embryos that have been treated with either radiation or cyclophosphamide to ablate endogenous bursal lymphoid cells. Remarkably, while embryo bone marrow contains functional pre-bursal cells, the bone marrow after hatching does not (Weber and Foglia, 1980). In contrast, pre-thymic

cells can be demonstrated in both the embryo and post-hatch bone marrow. In effect, this means that the source of cells colonizing the bursa is only present in the embryo and the bursa is therefore colonized in a single wave during embryonic development.

Colonization of Lymphoid Follicles in the Bursa

While the colonization of the bursal mesenchyme is likely selective for B cell progenitors, independent of whether they express cell sIg, there is a second and crucial level of selection that occurs during the colonization of lymphoid follicles in the bursa. Bursal follicles develop from the migration of B cell progenitors across the basement membrane that separates the mesenchyme from the bursal epithelium. Once these precursors migrate across they begin to proliferate and this proliferation drives follicle formation. We will therefore consider our understanding of the requirements for translocation across the basement membrane, before discussing the proliferation of bursal cells after they have translocated.

It has been clear for a number of years that while B cell progenitors containing non-productive rearrangements can be isolated from the embryo bursa, bursal cells that are clonally expanding in bursal follicles contain only productive rearrangements and express cell sIg (Reynaud *et al*., 1987; Thompson and Neiman, 1987; McCormack *et al*., 1989a). As a consequence, there was a widespread belief that sIg expression represented a critical checkpoint such that those B cell progenitors expressing sIg translocated across the basement membrane, whereas those B cell progenitors that did not express sIg did not translocate. This would be consistent with the observation that productive rearrangements were selected during embryonic life in the bursa, but not in embryo spleen (McCormack *et al*., 1989b). Several lines of evidence now support this contention, as will be discussed in more detail below.

The sIg-receptor complex on B cells (BCR) includes the Ig heavy and light chains non-covalently associated with the Igα/β heterodimer. As B cells develop, the first isotype of Ig expressed at the B cell surface is IgM. sIgM includes two μ heavy chains, held together by disulphide bonds, each covalently associated with a light chain again through disulphide bonds. The $(\mu L)_2$ tetramer represents the antigen binding portion of the sIgM receptor. The receptor is held in the plasma membrane by a transmembrane region that is in general hydrophobic. The cytoplasmic domain of chicken sIgM is only three amino acids in length, as is the case in other species. As a consequence, this domain is too short to couple the sIgM receptor to the signalling molecules responsible for signal transduction downstream of sIgM and signalling through sIgM is a property of the Igα/β heterodimer. Both Igα and Igβ contain an extracellular Ig-like domain, a transmembrane region and a cytoplasmic tail that contains conserved motifs implicated in signal transduction. Igα and Igβ are covalently bound to one another by means of an extracellular disulphide bond formed close to the plasma membrane. In chicken B cells the stoichiometry of the association of Igα/β with the $(\mu L)_2$ sIgM molecule is unclear. In murine B cells, however, there is evidence suggesting that one Igα/β heterodimer is associated with each $(\mu L)_2$ tetramer. At this point there is no reason to expect the chicken BCR complex to be fundamentally different from the murine BCR complex. The signal transduction pathways downstream of the BCR complex have been explored in detail in mammalian B cells and again there is no reason to expect fundamental differences between mammalian and avian BCR signalling. A detailed analysis of BCR signalling is beyond the scope of this chapter, but the reader is referred to other recent reviews and references contained therein (Sayegh *et al*., 2000; Pike *et al*., 2004a).

In murine cells, expression of μ, light chain, Igα and Igβ are all required to support the expression of the sIgM receptor complex at the cell surface. In the absence of any one of these polypeptides, the remaining chains are held up in the endoplasmic reticulum and degraded (Matsuuchi *et al*., 1992). In chicken cells, the $(\mu L)_2$ receptor can be expressed at the cell surface in the absence of Igα/β but without the signalling properties of Igα/β the $(\mu L)_2$ receptor fails to transduce signals and can be considered biologically inert (Demaries and Ratcliffe, 1998). Molecular cloning of the components of the chicken BCR complex has allowed a direct assessment of the requirements

for sIgM expression in supporting the functional colonization of bursal follicles in the developing chick embryo.

Since chicken Ig gene rearrangement generates minimal diversity, there has been considerable discussion as to whether the chicken sIg receptor generated by rearrangement could recognize a ligand in the bursal microenvironment. This argument was based on the analogy between the mammalian pre-BCR, which contains the non-variable VpreB and λ5 chains, and the chicken sIg receptor generated by rearrangement. In this model, recognition of a bursal ligand as a consequence of sIg expression would be required for sIgM transduced signalling leading to support of further B cell development. Since this could only occur in the context of functional rearrangement of Ig heavy and light chains, this model would explain how the colonization of lymphoid follicles in the bursa could be limited to those bursal cells with functional BCR expression.

This issue was directly addressed using retrovirally mediated gene transfer *in vivo*. In these experiments a truncated form of the Ig μ heavy chain was introduced into the RCAS retrovirus, a productive retroviral vector that can infect developing B cell precursors *in vivo*. The truncated μ(Tμ) construct contained a deletion of the V_H and Cμ1 domains and so did not associate with the light chain, nor required the presence of light chain for surface expression. Nonetheless, when expressed in B cells, this construct associated with the Igα/β heterodimer resulting in expression of a Tμ complex that retains the capacity to transmit signals when appropriately stimulated by crosslinking. Infection of 3 EID chick embryos with the RCAS-Tμ construct resulted in the presence of bursal cells in the neonatal chick that expressed the Tμ receptor in the absence of endogenous sIgM. This formally demonstrated that recognition of a bursal ligand by the variable region of the sIgM receptor complex is not required to support the progression of B cell development. Nonetheless, since all bursal cells in such chicks expressed either endogenous sIgM or Tμ construct, these experiments provided evidence that some form of BCR complex expression is required for the normal progression of B cell development (Sayegh *et al*., 1999a).

Nonetheless, the requirement for expression of the Tμ receptor complex in these experiments left open the possibility that a ligand in the bursal microenvironment could still recognize, either the residual constant region domains of the Tμ chain itself or the extracellular domains of Igα and/or Igβ. These domains would either be expressed on the surface of bursal B cells expressing the Tμ receptor construct or endogenous sIgM, consistent with the observed results. To address this possibility, chimaeric constructs were generated in which the extracellular and transmembrane domains of murine CD8α and CD8β were fused to the cytoplasmic domains of chicken Igα and Igβ, respectively. Co-expression of CD8α and CD8β preferentially leads to the formation of CD8α/β heterodimers. As a consequence, the cytoplasmic side of the chimaeric receptor complex mimics the sIgM receptor, with the cytoplasmic domains of Igα and Igβ, whereas the extracellular portion of the receptor does not contain any domains associated with either the $(\mu L)_2$ receptor or its associated Igα/β heterodimer.

Co-infection of B cell precursors in developing chick embryos with viruses encoding CD8α:Igα and CD8β:Igβ fusion proteins resulted in the development of bursal B cells expressing the chimaeric CD8α/β heterodimer in the absence of endogenous sIg expression. Thus even under circumstances where no extracellular domains of the sIgM receptor complex were expressed, expression of the cytoplasmic domains of Igα and Igβ was sufficient to support the normal progression of B cell development. More detailed analysis demonstrated that membrane proximal expression of the cytoplasmic domain of Igα was necessary and sufficient to support B cell development (Pike *et al*., 2004b).

The cytoplasmic domains of Igα and Igβ include three and two conserved tyrosine residues, respectively. The two tyrosine residues of Igβ comprise an immunoreceptor tyrosine-based activation motif (ITAM). Similarly the two membrane proximal tyrosine residues of Igα also comprise an ITAM with the third tyrosine residue forming a putative binding site for the adaptor protein BLNK. Based on results derived from site-directed mutagenesis of the CD8:Igα/β chimaeric receptors, support for B cell development requires the presence of the Igα ITAM combined with a third

tyrosine residue that can be derived, either from the third tyrosine of the Igα cytoplasmic domain or either of the two tyrosines of the Igβ cytoplasmic domain (Pike and Ratcliffe, 2005).

The importance of signalling motifs, in supporting the colonization of bursal follicles with B cell precursors expressing the CD8 chimaeric receptors, firmly implicates biochemical activity as critically important for developing B cells to sense the expression of the functional BCR complex. Nonetheless, since these signals can be provided by receptor constructs that have no Ig complex-related domains in the extracellular region, the notion of basal signalling as sufficient to support B cell development has been developed. Surface receptors such as the BCR complex are constantly being phosphorylated, even under conditions where they are not being actively stimulated. This is a consequence of low-level constitutive kinase activity found in all cells. Phosphorylated receptors are also constantly being dephosphorylated by phosphatases that also have significant constitutive activity. The levels of BCR phosphorylation in unstimulated B cells is therefore a consequence of the balance between receptor phosphorylation and dephosphorylation. The net result of these competing processes is that unstimulated B cells will contain a small, but significant, proportion of phosphorylated BCR complexes at any given point in time that have the capacity to couple to downstream signalling pathways. Thus BCR expression, even in the absence of ligation, will lead to a certain level of downstream signalling. At this point, the precise signalling pathways required for the progression of bursal B cell development remain unclear (Pike and Ratcliffe, 2005).

Colonization of bursal follicles by B cell precursors expressing functional BCR complexes represents an essential checkpoint in early B cell development. This colonization involves the migration of cells across the basement membrane that separates the mesenchyme from the bursal epithelium. Based on microscopic examination, however, there is evidence that the first cells which migrate across the basement membrane are not lymphoid cells, but rather have been described as secretory dendritic cells (Olah and Glick, 1978; Olah *et al*., 1979). These cells may be responsible for providing the chemokines or other molecules that attract the appropriate B cell precursors across the basement membrane (see also Chapter 2). Under these circumstances, one would expect the secretory dendritic cells to establish a chemokine gradient. The other prediction of such a model would be that only those B cell precursors, which had undergone productive rearrangement, would be responsive to such a chemokines gradient. Thus the basal signalling that occurs following productive sIg expression might in turn lead to the expression or up-regulation of receptors specific for particular chemokines.

Analysis of parabions in which blood-borne precursors could arise from either one of two joined embryos allowed an assessment of the number of pre-bursal cells that colonize each bursal follicle. Thus under circumstances where the bursal mesenchyme contained precursors from both “strain A” and “strain B”, a significant proportion of follicles contained exclusively strain A or strain B bursal cells, while other follicles contained a mixture of strain A and strain B cells. This demonstrates that each follicle is colonized by a small number of precursors and the clearest estimates have put this number at between 2 and 5 with an average of about 3 (Pink *et al*., 1985). Since follicles are colonized by cells expressing sIg, and since sIg is allelically excluded, the distribution of cells expressing one or other IgM allotype in normal allotype heterozygous chicks also provides an estimate of the number of B cell precursors per follicle. Again a range of 2–5 with an average of about 3 precursors per follicle was consistently observed (Ratcliffe *et al*., 1986).

There are about 10 000 follicles in the average bursa, which, consequently, means that the entire B cell compartment in the chicken is derived from about 30 000 precursors that have undergone productive rearrangement at the heavy and light chain loci. Since only one in three light chain rearrangements are in frame and only one in nine heavy chain rearrangements are productive, only about 3% (i.e. 1 in 27) of B cell precursors that have undergone rearrangement will contain both functional heavy and light chains. Therefore, from theoretical considerations, this places the minimum number of B cell precursors in the developing embryo at approximately 10^6. This is similar to the number of B cell precursors that have been identified by quantitative PCR in the developing embryo (Reynaud *et al*., 1992).

Growth of Bursal B Cells in Bursal Follicles

Following the migration of sIg expressing cells into bursal follicles, there is a rapid induction of proliferation among these cells. This proliferation coincides with the induction of gene conversion resulting in the diversification of the VDJ_H and VJ_L sequences leading to the generation of a diversified repertoire of B cell specificities. At this point the molecule(s) responsible for inducing this rapid proliferation are not known. The chicken homologue (chBAFF) of the mammalian cytokine B cell activating factor (BAFF) has been cloned and shown to be expressed at high levels in the chicken bursa (Koskela *et al*., 2004; Schneider *et al*., 2004). While chBAFF is therefore a candidate for the molecule responsible for driving the proliferation of bursal B cells and has been shown to support some bursal cell proliferation, this would represent a clear distinction in function between chicken and mammalian BAFF. Typically mammalian BAFF is considered not to be an inducer of B cell proliferation, but rather to be a cytokine that maintains peripheral B cell viability and therefore to have a greater effect on B cell lifespan (Kalled, 2005). Interestingly, duck BAFF has been shown to enhance the proliferation of B cells induced with anti-IgM antibodies (Guan *et al*., 2006) suggesting a possible mechanism for differential growth of B cell in the bursa depending on whether they have encountered antigen. Nonetheless, at this point it is likely that the major driving force behind the extensive B cell division seen in bursal follicles has yet to be defined.

During the final week of embryonic development, the number of sIg-expressing B cells in the bursa increases exponentially (Lydyard *et al*., 1976; Reynolds, 1987). During this time, bursal cells typically undergo cell division approximately every 10 h and while there is some cell death among bursal cells at this stage, the amount of cell death is substantially less than what is seen in the bursa after hatching.

At this point, we can consider the role of bursal B cell development in the embryo as an expansionary phase in which the bursal B cell compartment goes from about 30 000 cells, with a very limited repertoire, to approximately 10^7 cells with a highly diversified repertoire. The bursal microenvironment is absolutely crucial for the induction of gene conversion. This has been best demonstrated in chicks that have been "tailectomized" at 3 EID. This procedure removes the tissue destined to become the bursa and so the bursal rudiment fails to develop. Remarkably in such chickens, relatively normal numbers of B cells develop in the periphery suggesting that in the absence of the bursa, there are alternative sites in which B cells expressing sIg can develop (Jalkanen *et al*., 1983, 1984). Crucially however, B cells developing in the absence of the bursa show a very restricted repertoire and their V genes do not contain significant levels of gene conversion events (Mansikka *et al*., 1990).

Development of the Bursa After Hatching

By the time of hatching, the bursa contains about 10 000 follicles, each of which contains about 1000 bursal B cells. Each follicle is directly connected to the bursal epithelium which separates the lymphoid compartment from the bursal lumen and the bursal lumen is itself connected to the gut lumen by the bursal duct (fully described in Chapter 2). Shortly after hatching the bursa goes through a number of changes that strongly suggest its function may also change.

The first change in bursal structure is the modification of the bursal epithelium overlying each follicle resulting in the generation of "epithelial tufts" (Ackerman and Knouff, 1959). These structures are highly specialized and function to transport the content of the bursal lumen into the lymphoid compartment of the bursa. Since the bursal lumen is connected to the gut lumen, this provides a means by which bursal B cell development can continue in direct contact with molecules derived from the gut. In this regard, the cells of the bursal epithelial tuft appear to be structurally and functionally analogous to the M cells of mammalian appendix or Peyer's patch (Bockman and Cooper, 1973; see also Chapter 2). The other major change that begins to occur at around the time of hatching is the segregation of bursal follicles into defined cortical and medullary regions. Several lines of evidence suggest that these events are interconnected, as will be discussed below (Fig. 4.3).

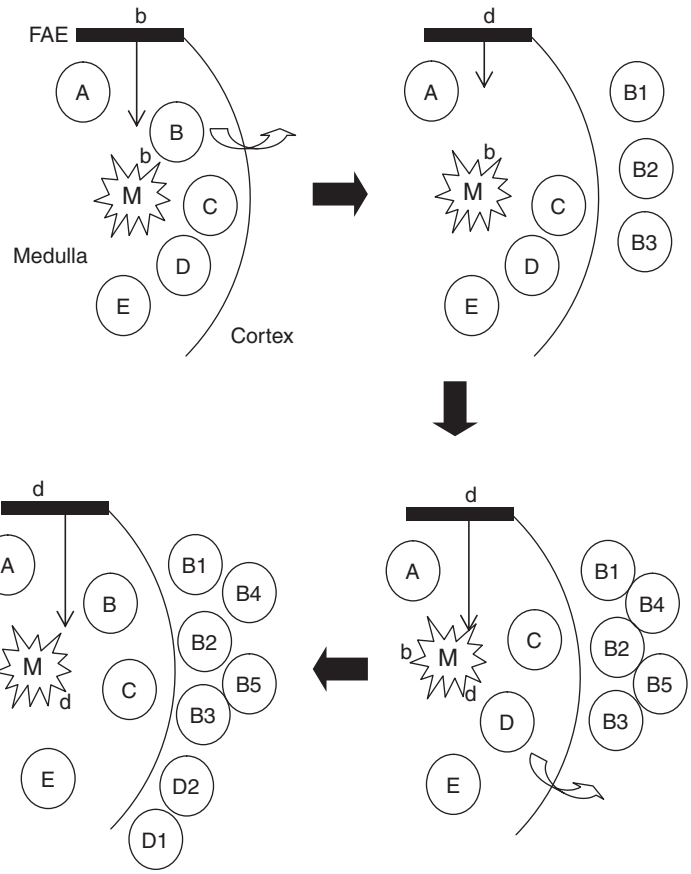

FIGURE 4.3 A model for antigen driven cortico-medullary redistribution of chicken B cells. Antigen labelled b entering the bursal medulla through the follicle-associated epithelium (FAE) or epithelial tuft is picked up and presented by dendritic cells. Presentation of antigen b to medullary cells specific for antigen b (labelled B) induces migration of the specific cell population to the cortex (top left). In the cortex these cells proliferate and continue to diversify their Ig genes by gene conversion indicated as B1, B2, B3, etc. In the meantime, the antigenic environment in the gut may change leading to the uptake of antigen d (top right). Antigen d is picked up and presented by dendritic cells, where it is recognized by "d" specific bursal cells, leading to their migration into the cortex (bottom right).

Prior to hatching, bursal follicles are rather homogeneous with rapidly dividing B cells interspersed among a network of stromal cells. There is no clearly defined sub-structure to the follicle. Starting around the time of hatching, however, some B lineage cells migrate back across the basement membrane and begin to proliferate between the basement membrane and the interconnective tissue that separates one follicle from another. Once the cortical and medullary regions have become segregated from each other, rapid bursal B cell proliferation continues in the cortex but not in the medulla. Stathmokinetic analyses indicate that the rate of cell division in the cortex of bursal follicles is similar to what is seen in the embryo bursa, whereas the rate of cell division in the bursal medulla is reduced by at least an order of magnitude (Reynolds, 1987). This provides a strong indication the medullary and cortical regions of the bursa likely have functional, as well as structural, differences.

Several lines of evidence have suggested that the redistribution of bursal follicular B cells into cortical and medullary compartments is regulated, in a fundamentally different way, from the initial colonization of bursal follicles in the embryo. If bursal rudiments are grafted into the abdominal wall of normal embryo recipients, then they become colonized by B cell precursors derived from the recipient embryo. Follicles develop normally during embryonic life, but the engrafted bursa does not develop normally after hatching (Houssaint *et al*., 1983). Similarly, if the

bursal duct is ligated during embryonic development, thereby precluding the normal traffic of gut-derived molecules into the bursal lumen, bursal follicle development is normal in the embryo, but the bursal cortico-medullary structure fails to develop normally after hatching (Ekino *et al*., 1980; Ekino, 1993). Taken together these observations suggest that exposure to gut-derived molecules is critical for the normal development of the cortico-medullary structure of the bursa after hatching.

In principle, these gut-derived molecules could be bacterially derived mitogens directly inducing maturation and proliferation of bursal B cells or bacterially derived products inducing stromal cells, perhaps via Toll-like receptor signalling, to produce cytokines supporting B cell development. Alternatively, bacterial- or gut-derived products could function as superantigens acting polyclonally on developing B cells, or as specific antigens binding to bursal cell sIg. Any of these situations would lead to a requirement for gut-derived products to access the lymphoid compartment of the bursa, thereby explaining the importance of the connection to the gut lumen and the development of the epithelial tufts.

Experiments in which the development of B cells has been driven by retroviral transduction of either the Tμ chain or CD8α:Igα chimaeric receptors have, however, yielded some insights into how the later stages of B cell development might be regulated. Either the Tμ receptor or the CD8α:Igα receptor supported all the early stages of early B cell development, including colonization of bursal follicles, rapid oligoclonal expansion of B cells within the bursal follicles and the induction of gene conversion (Sayegh *et al*., 1999a; Pike *et al*., 2004b). In addition, the cell-surface phenotype of bursal cells supported by either Tμ or CD8α:Igα receptors was indistinguishable from the phenotype of normal bursal cells from the same stage of development. As a consequence, it would seem unlikely that those bursal cells supported by Tμ or CD8α:Igα receptors would selectively lack the capacity to respond to a polyclonal signal that was independent of sIg expression. Nonetheless, neither the Tμ receptor (Sayegh and Ratcliffe, 2000) nor the CD8α:Igα receptor (Aliahmad *et al*., 2005) supported B cell development after hatch. This in turn means that the development of B cells after hatching is unlikely to be driven by either bacterially derived polyclonal mitogens acting directly on the bursal B cells themselves, or by the induction of stromal cell-derived cytokines supporting B cell development in the absence of sIg binding.

Conversely, these results support a model in which the development of B cells after hatching is dependent on the presence of Ig V region sequences on the bursal B cell surface. This in turn would suggest that sIg receptor ligation as distinct from receptor expression is required for the later stages of B cell development. One could envisage two potential explanations for this observation. One possibility is that the bacterial milieu of the gut contains superantigens which bind B cell sIg independent of the specificity of the individual receptors. There is some precedent for B cell superantigens recognizing particular V_H gene families in mammalian B cells (Silverman, 1992; Pospisil *et al*., 1995), but to this point there is no evidence for this occurring in the chicken and candidate superantigens have not been identified.

An alternative possibility is based on the probable antigenic heterogeneity of molecules present in the gut and therefore being transported into the lymphoid follicles. By the time this transport is initiated, several days after hatching, there is substantial diversity of B cell sIg receptors and so it is likely that in each follicle there will be at least some bursal B cells that have Ig receptors specific for molecules transported in from the gut. If sIg receptor ligation is required for the later stages of B cell development, it follows that at any given point in time there would likely be some B cells in each follicle with the appropriate specificity to recognize gut-derived antigens. This recognition may be sufficient to support the cortico-medullary redistribution of bursal cells after hatching. There is some evidence supporting this possibility. In chicks transduced with the CD8α:Igα chimaeric receptor, intrabursal introduction of anti-CD8α antibodies maintained the presence of cells expressing CD8α:Igα after hatching, consistent with a requirement for sIg receptor ligation for B cell development after hatch (Aliahmad *et al*., 2005). Similarly, duck bursal B cells stimulated with anti-IgM antibodies can be induced to proliferate in the presence of duck BAFF (Guan *et al*., 2006).

After hatching, the rate of growth of the bursa slows, despite the fact that B cells in the cortex of bursal follicles are rapidly dividing (Reynolds, 1987). Based on direct analyses of the rates of

cell death among bursal lymphocytes and cell emigration from the bursa to the periphery it has been estimated that about 95% of newly generated bursal cells die *in situ* by apoptosis (Lassila, 1989; Motyka and Reynolds, 1991). Although it has been demonstrated that loss of cell sIg precedes the induction of apoptosis (Paramithiotis *et al*., 1995), it still remains unclear why such a high proportion of bursal cells die *in situ*. In mammalian B cell development extensive cell death results from the accumulation of non-productive rearrangements. Cells that fail to express a sIg receptor die. This cannot be the situation in the bursa since all follicular B cells are derived from cells that express sIg. One alternative possibility that has been addressed is that the process of gene conversion might be highly error prone, leading to frequent out of frame V gene sequences. However in B cells supported by the expression of the Tμ receptor, an analysis of gene conversion events revealed an error rate of no more than 2–3%, even under circumstances where non-functional gene conversion events would not be selected against (Sayegh *et al*., 1999b).

A further possibility is that although gene conversion might generate predominantly in frame products, combinations of heavy and light chain V regions could potentially be generated that fail to pair to form a functional Ig molecule. Evidence supporting this possibility comes from an analysis in which random combinations of full length μ heavy chains and light chains were transfected into chick embryo fibroblasts. Surprisingly only about one-half of these combinations resulted in high-level sIg expression, with many other combinations failing to be expressed at the cell surface (Neschadim and Ratcliffe, manuscript in preparation). As a consequence, even though gene conversion may not be intrinsically error prone, a substantial proportion of cells that are undergoing gene conversion may generate V_H/V_L combinations that fail to be expressed at the cell surface leading to apoptotic elimination of such cells. This may contribute to the high levels of cell death in the bursa.

While there is clearly trafficking of bursal cells from the follicular medulla to the cortex, it is not clear whether this trafficking is continuous, or the cortex, once established, is simply maintained by proliferation of cortical cells. Nonetheless, the maintenance of antigen in the medulla of bursal follicles at least suggests the possibility that the medulla to cortex trafficking of bursal cells may continue indefinitely. This in turn might suggest that once a bursal cell has migrated into the follicular cortex, it might undergo a finite number of cell divisions prior to either emigrating to the periphery or undergoing cell death.

Development of Peripheral B Cell Populations

The function of the bursa is to generate naïve B cells for export into the periphery. Introducing the fluorescent dye fluorescein isothiocyanate (FITC) into the bursal lumen results in its uptake into bursal follicles and *in situ* labelling of bursal cells (Lassila, 1989; Paramithiotis and Ratcliffe, 1993, 1994a, b). As labelled cells emigrate from the bursa to the periphery, they can be detected by their fluorescence and defined as recent bursal emigrants. Direct quantitation of the number of recent bursal emigrants in the periphery indicated that the hourly emigration rate of B cells corresponded to approximately 1% of the peripheral blood B cell population. This is consistent with 5% of newly generated bursal B cells emigrating to the periphery.

In the juvenile bursa most of the cell proliferation is occurring in the follicular cortex. By analysis of bromodeoxyuridine incorporation following rapid uptake of label into the follicular cortex, bromodeoxyuridine-labelled cells were observed in the peripheral blood prior to their appearance in the follicular medulla (Paramithiotis and Ratcliffe, 1994a, b). This indicates that most recent bursal emigrants in peripheral blood are directly derived from the bursal cortex.

The lifespan of peripheral blood B cell populations has been assessed following surgical removal of the bursa. At 3 weeks of age, approximately 60% of peripheral blood B cells disappear with an average lifespan of about 3 days. This accounts for the great majority of bursal cell emigration from the bursa. The LT2 antigen, defined by the LT2 monoclonal antibody, stains about 60% of peripheral blood B cells from 3-week-old chicks. Three days after surgical bursectomy the frequency of LT2-expressing bursal cells is reduced to less than 1%. This indicates that the short-lived population of recent bursal emigrants expresses the LT2 antigen (Paramithiotis and Ratcliffe,

1996). Although the LT2 antigen is a convenient marker of this short-lived B cell population in the periphery, the LT2 antigen itself has not been defined (Fig. 4.4).

Following the initial loss of the short-lived B cell population after surgical bursectomy a further 30–35% of B cells disappear from the peripheral blood with a lifespan of about 3 weeks. These cells do not express the LT2 antigen and therefore represent a functionally and phenotypically distinct population of peripheral blood B cells (Paramithiotis and Ratcliffe, 1993, 1994a, b, 1996). Analysis of the distribution of LT2 expression on FITC labelled recent bursal emigrants indicated that about 90% of emigrants expressed the LT2 antigen whereas about 10% of recent emigrants were $LT2^-$. This is an important observation as it suggests that functional differences between short- and longer-lived B cells in the periphery is established in the bursa and that B cells emigrate from the bursa already committed to being short- or longer-lived B cells. This suggests that at least two distinct pathways of B cell maturation must exist in the bursa.

Most bursal emigrants come from the follicular cortex and express the LT2 antigen, which is predominantly expressed by bursal cells in the follicular cortex. Since the accumulation of gut-derived antigens in the bursa is restricted to the follicular medulla, this population of short-lived cells might represent a naïve repertoire of B cell specificities. In contrast, approximately 10% of bursal emigrants do not express the LT2 antigen and likely represent the precursors of the population of longer-lived peripheral blood B cells. The source of these cells within the bursa is unclear although, based on the distribution of bromodeoxyuridine labelling in pulse chase experiments, there is some evidence that some bursal cells which have undergone division in the follicular cortex can ultimately migrate to the follicular medulla. Since the follicular medulla contains gut-derived antigens, it is tempting to speculate that the longer-lived population of peripheral blood B cells represents a population that has been selected by exposure to antigen in the follicular

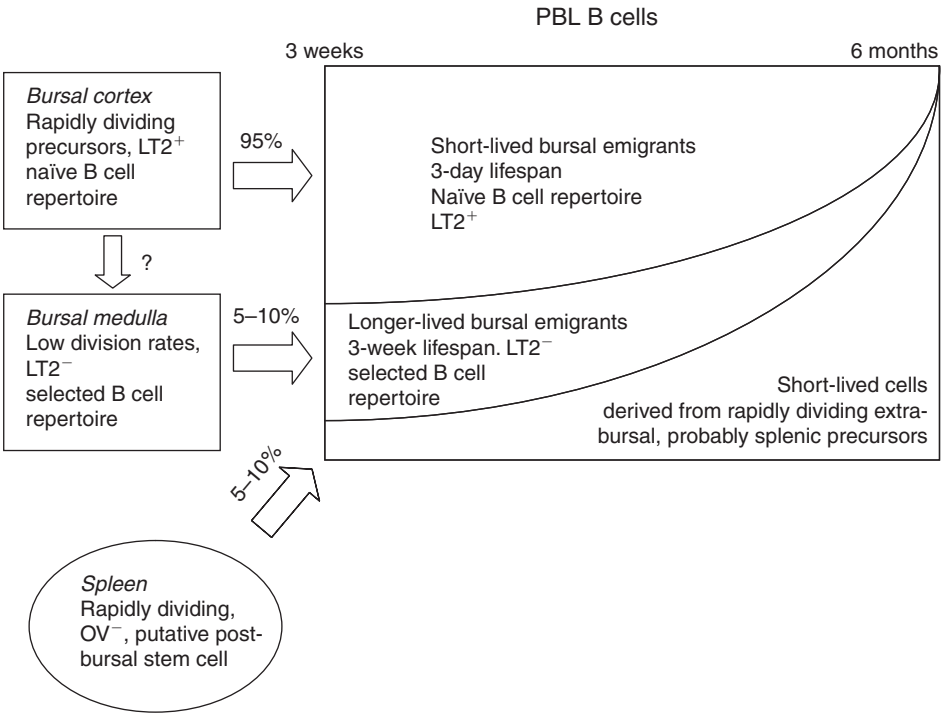

FIGURE 4.4 Heterogeneity of peripheral blood B cells in the chicken. There are three populations of peripheral blood B cells from potentially distinct sources in the peripheral blood. The indicated percentages represent the approximate proportions of input cells and the vertical division of the peripheral blood B cell population indicates relative proportions of the three populations in peripheral blood as a function of time.

medulla. The combination of short- and longer-lived populations of B cell in the periphery might therefore represent a combination of (1) rapidly turning over and constantly replenished B cells, with random specificities and (2) longer-lived B cells selected for a repertoire, defined by the range of antigens present in the bursa and therefore present in the environment.

Approximately, 4–5 weeks after surgical bursectomy the number of residual B cells in the peripheral blood stabilizes at about 5% of the original B cell population. This level of B cells remains constant thereafter and, although they are not undergoing cell division in the peripheral blood itself, their rapid uptake of bromodeoxyuridine suggests that they are constantly being derived from rapidly dividing precursor cells (Paramithiotis and Ratcliffe, 1993). Since this is observed in chickens in which the bursa has been ablated, the source of these cells must be extra-bursal. Therefore in addition to the short- and longer-lived populations of bursal emigrants that are non-dividing in the periphery, the peripheral blood contains a third population of B cells that are short-lived cells derived from rapidly dividing extra-bursal precursors. The proportion of this last population is about 5% of peripheral blood B cells at about 3 weeks of age. However, the frequency of this population appears to change with age. If the bursal is surgically removed earlier than 3 weeks of age, the frequency of this population decreases, whereas if the bursa is removed later in life, the frequency of this population increases. Therefore, even though the immediate precursors to this population of B cells are extra-bursal, this precursor population must ultimately be derived from the bursa.

This third population of peripheral blood B cells may also represent the source of peripheral blood B cells in the older chicken since the bursa involutes by about 6 months of age. The most likely source for these cells is the spleen. It has long been known that the spleen contains a population of cells that have been functionally defined as post-bursal stem cells with the capacity to provide long-term reconstitution when transferred into cyclophosphamide-treated recipients. In the normal spleen most B cells express the OV alloantigen, however there is a small population of B cells that lack expression of the OV antigen and are characteristically larger than the majority of splenic B cells. Following surgical bursectomy in 3-week-old chickens, there is a loss of splenic B cells, in parallel with the loss of peripheral blood B cells. However, those splenic B cells that lack the expression of the OV antigen are not reduced and can become the major population of residual splenic B cells. These cells, therefore, provide a strong candidate population for the post-bursal stem cell population, and a likely source of the population of peripheral blood B cells that are not sensitive to surgical bursectomy (Paramithiotis and Ratcliffe, 1994a, b).

Activation of Peripheral B Cells

The molecular mechanisms underlying the activation of murine B cells have been well defined. Antigen picked up by surface antigen receptors is processed, and antigen-derived peptides are presented in association with major histocompatibility (MHC) class II molecules on the B cell surface. Helper T cells specific for these peptide/MHC class II complexes interact directly with the responding T cells and provide help through CD40L on the T cell interacting with CD40 on the B cell surface. Implicit in this is the requirement for an MHC restricted interaction between the helper T cell and responding B cell. The naïve B cell is therefore activated by a combination of two signals, one through sIg binding antigen and the second through CD40 binding CD40L. This activates the responding B cell to the point where it can respond to cytokines, inducing proliferation and maturation to Ig secretion (reviewed in Mills and Cambier, 2003 and references therein).

At this point, the activation of chicken B cells to antibody secretion appears to be indistinguishable from the activation of mammalian B cells. Cyclophosphamide treatment destroys the lymphoid compartment of the bursa. Transfer of bursal cells into cyclophosphamide-treated recipients results in reconstitution of the bursal lymphoid population by donor-derived cells which can mature and reconstitute the periphery. The colonization of the bursa by donor cells is independent of the strain of donor cells and since recolonization is typically done within a few days of hatching, allogeneic donor cells are not rejected. Following bursal and subsequent peripheral reconstitution, both syngeneic and allogeneic B cells can be activated by polyclonal B cell activators

demonstrating functional reconstitution by both sources of bursal cells. In contrast, only B cells sharing MHC class II with the host helper T cell population are activated by T cell dependent antigens, providing direct evidence that an MHC class II restricted interaction between the helper T cell and responding B cell is required for chicken B cell activation (Vainio *et al.*, 1984).

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5

AVIAN T CELLS: ANTIGEN RECOGNITION AND LINEAGES

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INTRODUCTION
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INTRODUCTION

The antigen recognition by T cells is a highly sophisticated and remarkable process mediated by the T cell receptor (TCR). Two key features distinguish T cell antigen recognition from most surface receptors that are pre-committed to a specific ligand. The TCR is randomly generated and it recognizes a set of diverse peptides complexed to self major histocompatibility complex (MHC) molecules at a density of only 0.1–1 molecules/μm^2.

Most of the paradigms for T cell function have been established in man and mouse. Although birds and mammals evolved from a common ancestor more than 200 million years ago, many general aspects of mammalian T cell structure and function also apply to avian T lymphocytes. Nevertheless, studies on the chicken indicate some unique T cell features. Here, we will summarize various aspects of TCR structure and function and try to integrate some of the recent key findings concerning mammalian TCR.

TCR STRUCTURE AND LINEAGES

Chicken T cells use a heterodimeric surface receptor for antigen recognition. Each TCR chain is composed of two immunoglobulin (Ig) superfamily domains. The membrane distal variable (V) region is composed of a V-type Ig domain and shows extensive diversity. This diversity is generated by the process of somatic DNA recombination (Tonegawa, 1983). All TCR chains contain a single membrane proximal constant (C) domain – as opposed to up to four C domains in antibodies – which defines the isotype of the TCR chain. The V and C regions are anchored in the plasma membrane by a connecting peptide and a hydrophobic transmembrane region that has one or two basic residues. The TCR chains have very short cytoplasmic regions, which do not

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allow signal transduction. All jawed vertebrates have four distinct TCR chains, the α, β, γ and δ TCR. By analogy with the Ig molecule on B cells, that consists of two heavy and two light chains, the α and γ TCR and the β and δ TCR resemble light and heavy chains, respectively. A cysteine in the connecting peptide is used to form an intermolecular disulphide bridge between two of the chains to generate either the $\alpha\beta$ TCR or the $\gamma\delta$ TCR, which define the two major T cell lineages. Additional surface molecules are used to further divide T cells into the $CD4^+$ T helper population that binds to MHC class II molecules and $CD8^+$ cytotoxic T cell population that surveys MHC class I molecules. Additional T cell subsets are defined by their secretion of cytokines.

Somatic DNA Recombination

The Ig diversity of chicken B cells is generated by gene conversion, a process taking place during bursal development (Reynaud *et al*., 1985 and reviewed in Chapter 4 of this book). T cells develop from the precursors in the thymus. Details of chicken T cell ontogeny and thymic development are described in Chapter 3. Somatic DNA recombination creates variability in the TCR thus ensuring a sufficient repertoire. For a comprehensive description of the molecular events leading to somatic DNA recombination, the reader is referred to excellent reviews covering the subject (Fugmann *et al*., 2000; Gellert, 2002; Maizels, 2005; Jung *et al*., 2006). The principles of somatic DNA recombination in chicken and mammalian T cells are identical. The basic concept of somatic DNA recombination is that a reservoir of small gene fragments is randomly assembled during thymic development to form a complete gene encoding a TCR. Each TCR is therefore encoded by a cluster of genes located on different chromosomal regions. The TCR clusters possess a similar organization to a set of 5′V genes, followed by a number of diversity (D) genes in the case of heavy chains, and succeeded by joining (J) genes and one or few 3′ constant (C) genes. In each thymocyte, the TCR genes are successively activated to form productive TCR rearrangements. In the case of the α or γ TCR, a randomly selected V gene is combined with a J gene and for TCR heavy chains β and δ an initial DJ rearrangement is succeeded by a VDJ combination. The regions between the V, (D) and J genes are excised. The process of somatic DNA recombination is mediated by the conserved enzymes encoded by the recombinase-activating genes 1 and 2 (RAG1 and RAG2) that are directed by special recombination signal sequences located in the vicinity of each V, D and J gene. During transcription, the combined V(D)J genes are spliced to the 3′C gene to form a complete TCR gene. This random selection of genes from existing gene pools generates a large V repertoire, and combination of two chains to form a TCR heterodimer increases this diversity. The peptide MHC binding site is specially generated by protruding loops of the V domains, which are homologous to the Ig complementarity-determining regions (CDR) of antibodies and display extensive diversity. Whereas the CDR1 and CDR2 regions are encoded by the V genes, the CDR3 region is generated by the juxtaposition of V(D)J genes. During the process of somatic DNA recombination, the joining of gene fragments is imprecise and subjected to base deletions by exonuclease activity, non-encoded base insertions (N-nucleotide additions) and insertions of short palindromic sequences (P additions) such that up to nine non-template bases are added. The major enzymes mediating these processes, such as RAG and terminal deoxynucleotide transferase (TdT), have also been characterized in the chicken (Thompson, 1995; Yang *et al*., 1995). Thus, the CDR1 and CDR2 of the TCR are germline encoded, whereas the CDR3 is generated randomly due to base insertions and deletions and shows the highest degree of variability. Currently, 13 independent crystal structures are available for the mammalian TCR (Rudolph *et al*., 2006). Models of these TCR structures have shown that the CDR1 and CDR2 mainly contact the MHC α helices, and in the case of CDR1 also the end of the antigen peptide. The CDR3, however, is localized directly over the centre of the MHC peptide binding groove and makes contact with the peptide bound in the groove, as well as the MHC α helices (Bentley and Mariuzza, 1996; Davis *et al*., 1998). Receptor editing (Nemazee and Hogquist, 2003), a process where T cells continue to rearrange V and J segments located up or downstream of the initially rearranged VJ genes has not been completely analysed in the chicken, mainly due to the lack of clonal T cell lines. The diversity of T cells is fixed after thymic development and is not

changed after antigen recognition. This is different to B cells, which undergo somatic hypermutation in germinal centres after antigen recognition (Maizels, 2005). The organization of the individual chicken TCR clusters is described below.

Organization of the TCR Clusters

TCRα/δ Cluster

In mammals, the TCRα and TCRδ genes are encoded by a single cluster designated TCRα/δ. All non-mammalian TCRα/δ gene clusters characterized so far seem to share this configuration (Rast *et al*., 1997; Kubota *et al*., 1999; Litman *et al*., 1999). In this TCRα/δ cluster, the TCRδ encoding VDJC genes are located between the Vα and Jα sequences. Therefore, somatic recombination of the TCRα locus leads to the excision of the entire TCRδ cluster. This TCRδ deletion has been found on both alleles of all chicken αβ T cell lines tested, thus providing strong evidence for a conserved TCRα/δ locus (Kubota *et al*., 1999). Southern blot and sequence analyses indicate that there are two Vα families with a total of at least 25 members (Fig. 5.1; Göbel *et al*., 1994; Kubota *et al*., 1999). All T cell lines examined so far share a Vα member from one of these two families, suggesting that there are only two Vα families. These two Vα families share less than 30% amino acid homology. A cosmid clone representative of the 3′end of the TCRαδ locus has been sequenced and contains six Jα genes and a single Cα gene (Wang *et al*., 1997). Southern blot analysis suggests that there are more Jα sequences upstream of this region (Göbel *et al*., 1994). A homologue to the human apoptotic suppressor gene is located 6.3 kb downstream of the Cα gene in opposite transcriptional orientation (Wang *et al*., 1997). In mammalian thymocytes, the TCRβ chain first pairs with a TCRα homologue designated pTα; however, a pTα homologue has not been described in chicken. The four TCR C regions are each encoded by a single C gene in the chicken. This C gene is composed of four exons in the case of TCRα and TCRβ, while no information about the exact genomic organization is available for TCRγ and TCRδ (Wang *et al*., 1997; Shigeta *et al*., 2004). Unfortunately, apart from some short sequence similarities, TCR gene clusters have not been assembled in the draft sequence of the chicken genome. Therefore, there is no assignment to a chromosome, and the number of V, J and D genes is not clear in either case.

Embraced by the Vα and Jα genes, there is at least one large Vδ gene family, followed by two Dδ, two Jδ and a single Cδ gene (Kubota *et al*., 1999). This organization increases the combinatorial diversity, such that members of both Vα families can be utilized in conjunction with TCRδ genes. The Dδ elements may be utilized during the rearrangement with extensive variability in all possible combinations. So Vα and Vδ genes have been found to be rearranged directly to Jδ lacking

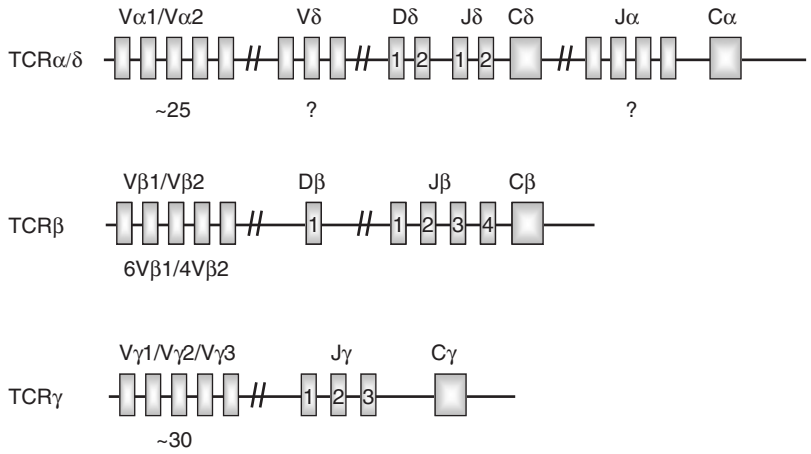

FIGURE 5.1 TCR gene loci. Schematic representation of the three chicken TCR loci. The number of genes is indicated where known. Note that distances between different elements are not known.

a Dδ gene or they were rearranged to either one of the two Dδ elements or alternatively to both Dδ genes in tandem. The Jδ genes were found to be equally represented in embryonic γδ T cells, whereas in adult γδ T cells there was a bias towards Jδ1 usage (Kubota *et al*., 1999).

TCRβ Cluster

The chicken TCRβ genes were the first non-mammalian TCR genes to be isolated (Tjoelker *et al*., 1990); a total of ten Vβ regions form the Vβ repertoire. Based on their nucleotide homology, these ten Vβ sequences are further divided into six Vβ1 and four Vβ2 family members (Fig. 5.1). Members within a family share over 90% homology, whereas the homology between the families is less than 40% (Lahti *et al*., 1991). As in mammals, the chicken TCRβ genes are intermingled with trypsinogen genes that can be divided into two multimember subfamilies, a trypsinogen I family with six members and a three-member trypsinogen II family (Wang *et al*., 1995). There is evidence that each Vβ1 and most Vβ2 genes are directly clustered with a trypsinogen I and trypsinogen II family member, respectively.

Two αβ TCR-specific monoclonal antibodies (mAb) have been generated (Cihak *et al*., 1988; Chen *et al*., 1989). The TCR2 mAb specifically recognizes all Vβ1 family members and the TCR3 mAb, the Vβ2 family members (Lahti *et al*., 1991; Chen *et al*., 1996). A single 14 nucleotide Dβ element is located downstream of the Vβ sequences (Tjoelker *et al*., 1990; Shigeta *et al*., 2004). The potential combinatorial diversity that results from only two Vβ families with high intrafamiliar homology and a single Dβ element seems to be reduced in comparison to the mammalian TCRβ cluster. The TCR V genes encode the CDR1 and CDR2 that are mainly responsible to contact the MHC α helices. The chicken MHC differs markedly from its mammalian counterpart because it is very compact with only few genes and it is less polymorphic (Kaufman *et al*., 1999; Chapter 8). It is therefore remarkable that the TCR contacting the MHC has also reduced germline diversity which seems to be sufficient to produce TCR that bind to all possible chicken MHC α helices. The CDR3 region contacting the MHC bound peptide, however, is generated by the Dβ element. All three reading frames of the Dβ are utilized and they encode a glycine that may be involved in the formation of the CDR3 loop (McCormack *et al*., 1991). The Dβ gene is followed by four Jβ sequences (Shigeta *et al*., 2004). Extensive N- and P-nucleotide additions as well as nucleotide deletions are observed at the junctions, thus creating a highly diversified CDR3. Due to the upregulation of the TdT, the extent of N-region addition increases during embryonic development. During rearrangement, the intervening chromosomal DNA is excised and forms so-called deletion circles that have also been detected for the TCRβ and TCRγ locus. These deletion circles are used as a marker for recent thymic emigrants (Kong *et al*., 1998, 1999). Moreover, as in mammals, germline transcripts of TCRβ were found before the detection of rearrangements, indicating that transcription of unrearranged TCR genes precedes the recombinase activity. Both VD and DJ rearrangements were detected at identical time points indicating that the chronology of rearrangement steps is not strictly regulated in the chicken (Dunon *et al*., 1994).

TCRγ Cluster

The TCRγ cluster consists of approximately 30 Vγgenes that can be grouped into three Vγ gene families with eight to ten members each (Fig. 5.1; Six *et al*., 1996). There are three Jγ genes and again a single Cγ. In mouse and man, different waves of γδ T cells with canonical VγJγ rearrangements during thymic development populate various tissues, such as Vγ3Jγ1Cγ1 T cells homing to the skin and Vγ5Jγ1Cγ1 present in the intestine. This situation is different in the chicken where as early as embryonic incubation day 10 of development members of all three Vγ families start to rearrange (Six *et al*., 1996). In addition, extensive junctional diversity is created by exonuclease activity, P- and N-region additions. The γδ TCR repertoire is therefore highly diversified from the beginning of rearrangement. The development of γδ T cells in the chicken is strictly thymus dependent. It has been demonstrated that intestinal and splenic γδ T cells are generated by two waves of thymic emigrants. While splenic γδ T cells are relatively short-lived, the intestinal γδ T cells derived from early thymic emigrants form a long-lived, self-containing population (Dunon *et al*., 1993, 1997).

CD3 SIGNALLING COMPLEX

Mammalian CD3

The CD3 molecules serve at least three functions. They are important during the assembly of the TCR complex, they control surface expression of the TCR complex by targeting incomplete complexes to degradation and they trigger signal transduction following TCR ligation. In mammals, the CD3 complex consists of CD3γ, CD3δ, CD3ε and a ζζ homodimer. The CD3 chains form two dimers, a CD3γ–CD3ε and a CD3δ–CD3ε heterodimer before assembly with the TCR. The extracellular domains of the CD3 molecules are composed of a single Ig-like domain, the transmembrane regions harbour one acidic amino acid that is important for the assembly and the intracellular domains contain an immunoreceptor tyrosine-based activation motif (ITAM) that is essential for signal transduction. The ζ chain has only nine extracellular amino acids, its transmembrane domain also contains an acidic residue and a cysteine for dimerization and the long cytoplasmic tail has three ITAM. Various aspects concerning assembly, structure and function of the mammalian CD3 complex have been summarized in excellent reviews (Klausner *et al*., 1990; Terhorst *et al*., 1995; Malissen *et al*., 1999; Malissen, 2003; Rudolph *et al*., 2006).

Chicken CD3γ/δ and CD3ε

In contrast to mammals, only two chicken CD3 genes exist, a CD3ε homologue and a CD3γδ gene with equal homology to mammalian CD3γ and CD3δ (Chen *et al*., 1986; Bernot and Auffray, 1991; Göbel and Fluri, 1997; Göbel and Bolliger, 2000). The genes encoding chicken CD3γ/δ and CD3ε are located on a 9.5 kb region on chromosome 24 and are flanked by two unrelated genes (ZW10 and EVA) (Göbel and Dangy, 2000). The precise genomic organization and the exon/intron structures are depicted in Fig. 5.2. Since the two CD3 genes in the chicken are tightly clustered and transcribed in opposite orientations, it is likely that both genes arose from an ancestral form by gene duplication. The exon/intron structures of all CD3 genes also argue in favour of this hypothesis. Although there are variations at both ends of the CD3ε and

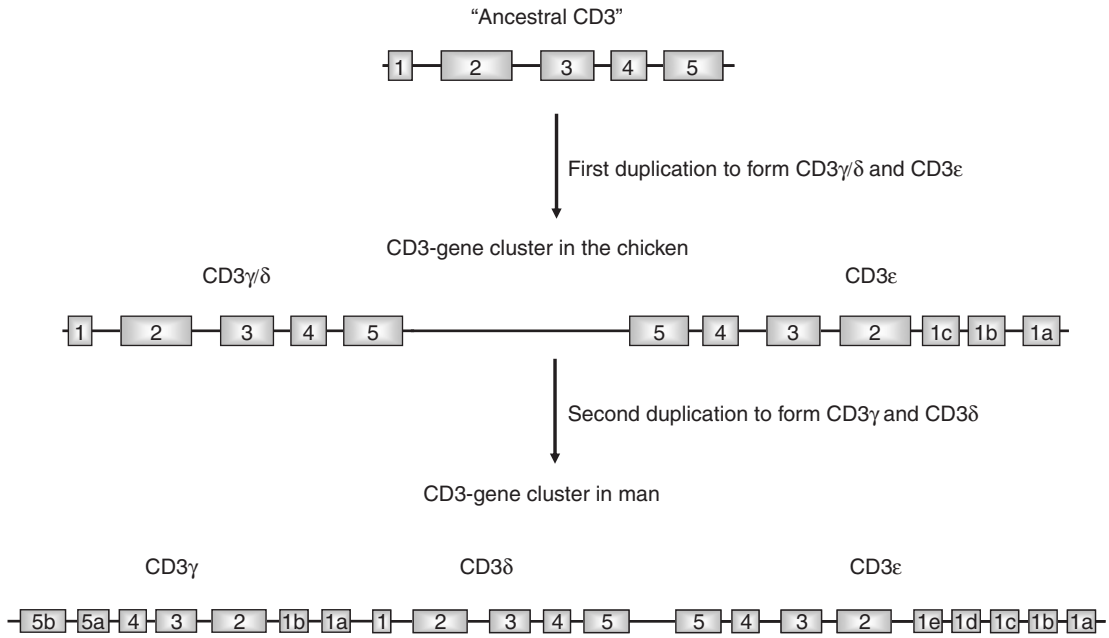

FIGURE 5.2 Model of CD3 gene evolution. Hypothetical model depicting the stepwise evolution of CD3 genes in vertebrates. The number of exons of each CD3 gene is shown. Sizes and distances are not drawn to scale.

mammalian CD3γ locus, their core structure has been preserved. We have therefore proposed that the three CD3 genes present in mammals arose by a stepwise evolution from an ancestral CD3 gene (Göbel and Bolliger, 2000; Göbel and Dangy, 2000). This CD3 ancestor was duplicated to form CD3γ/δ and CD3ε that are present in fish, amphibians and chickens (Dzialo and Cooper, 1997; Alabyev *et al*., 2000; Göbel *et al*., 2000; Park *et al*., 2001; Araki *et al*., 2005). Only in mammals has a second duplication taken place to form CD3γ and CD3δ. Interestingly, many features that have been described for either mammalian CD3γ or CD3δ are combined in the chicken CD3γ/δ. For example, certain sequence features depend on glycosylation and a cytoplasmic internalization motif (Göbel and Dangy, 2000).

ζζ Homodimer

In addition to the two CD3 genes, the ζζ homodimer is an essential part of the chicken TCR. The ζζ homodimer functions as an adaptor molecule that is used by various surface receptors including the TCR. It is not encoded in the same area of the CD3 genes, but on chicken chromosome 1. The importance of the ζζ homodimer as a signalling component is obvious by its short extracellular domain, and its long cytoplasmic region. The recent structural characterization of the ζζ homodimer has revealed that the importance of the two transmembranous aspartic acid residues is critical for the dimerization (Call *et al*., 2006). The cytoplasmic domain contains three repeats of the ITAM. An ITAM is defined as two YXXL motifs spaced by eight amino acids (Reth, 1989). The tyrosine residues of the ITAM become phosphorylated during cellular activation and mediate downstream signalling events (see below). The chicken ζζ homodimer has been so well conserved that it is able to associate with the mammalian TCR and to restore its function (Göbel and Bolliger, 1998). In mammalian TCR, an alternatively spliced version of the ζ chain designated η chain is sometimes assembled to form a ζη heterodimer; however, this has not been characterized in the chicken.

TCR Complex: Structural Models

The TCR complex consists of the TCR heterodimer and the signalling components. As stated above, all molecules (TCR heterodimer, CD3γ/δ, CD3ε and ζζ homodimer) are essential for surface expression. The valency (number of TCR heterodimers in a TCR complex) and the stoichiometry (molar ratios between subunits) is still unresolved and two models are proposed: a monovalent model with a single TCR heterodimer and a bivalent model consisting two TCR heterodimers. Moreover, no crystal structures of the entire complex exist. Recent biochemical studies, however, indicate that different multiunit receptor complexes on haematopoietic cells such as the killer inhibitory receptors, certain Fc and collagen receptors are assembled by similar mechanisms (Feng *et al*., 2005). All of these receptors are characterized by basic transmembrane residues in the ligand binding domain and acidic residues in the signalling dimer (Call and Wucherpfennig, 2005; Rudolph *et al*., 2006). These residues favour the formation of a three-helix interface composed of two acidic and one basic residue (Plate 5.1). This formation of trimers would imply that for the chicken a CD3γ/δ–CD3ε signalling dimer is associated with TCRα or TCRβ to form a TCRα–CD3γ/δ–CD3ε trimer and a TCRβ–CD3γδ–CD3ε trimer. In the following step, these two trimers (α–γ/δ–ε and β–γ/δ–ε) are assembled and the free basic charge on TCRα aids formation of a third trimer together with the ζζ dimer. Although the assembly of the chicken TCR complex has not been studied in great detail the conserved sequence features across species suggest similar assembly mechanisms. Apart from the transmembrane residues that are essential for the building of a complete TCR complex, the interactions of the extracellular Ig domains are important for proper assembly (Kuhns *et al*., 2006). Again the similarity between the chicken and mammalian TCR complex is remarkable: the TCR heterodimer has a 20–26 amino acid long connecting peptide, whereas the CD3 chains has only 5–10 amino acids. The CD3 chains all contain a highly conserved R(K)XCXXCXE motif close to the transmembrane region that has been implicated in the signalling of the TCR

complex (Kuhns *et al*., 2006) and forms a metal coordinated cluster (Sun *et al*., 2001). Despite these highly conserved sequence motifs it must be recognized that the overall protein homology between the chicken and mammalian TCR chains is very low. It is not surprising, therefore, that the chicken CD3 and TCR chains are not able to substitute for the respective mouse chains (Gouaillard *et al*., 2001). Notably, while all components of the TCR complex are essential for the surface expression of the αβ TCR, the murine γδ TCR contains only CD3γ–CD3ε dimers and lacks CD3δ (Hayes and Love, 2006). Information about the chicken γδ TCR is not available, though probably it also contains two CD3γ/δ–CD3ε heterodimers.

TCR Signal Transduction

Signal transduction has also been intensively studied in mouse T cells, but neglected in the T cells of the chicken. Because of strong conservation of intracellular signalling motifs, it can be predicted that signalling through the chicken TCR complex is similar to that of the mammalian complex. Central to the signalling capability of the TCR complex, following crosslinking by peptide MHC ligand, is signal amplification by ten cytoplasmic ITAM located in the cytoplasmic domains of the various CD3 chains and the ζζ homodimer. ITAM are not unique to the CD3 chains; they are utilized by many different cell surface receptors. As for the TCR complex, the ITAM in these molecules are not located in the cytoplasmic domain of the ligand binding receptor chain, but they are assembled into the complex by association with adaptor molecules such as CD3, ζζ homodimer, Fcγ chain or DAP-12. In all cases, the ITAM is encoded by two exons that are flanked by a type 0 intron, thereby forming a flexible two exon set. It has been speculated that this symmetric two exon ITAM set was used in many receptors as a signalling building block that could be inserted or deleted without disturbing the reading frame (Malissen, 2003).

The initial event taking place after ligand binding is mediated by a Src family kinase such as Lck. This Src kinase activity leads to the phosphorylation of the ITAM. Fully phosphorylated ITAM bind with high affinity to the tandemly arranged SH2 domains of ZAP-70, a member of the Syk family protein tyrosine kinases. ZAP-70 is also transphosphorylated by Lck, leading to an increase of its catalytic activity. Due to the presence of ten ITAM in the signalling units of one TCR complex, TCR triggering leads to signal amplification by recruiting up to ten ZAP-70 molecules. Moreover, several unique residues apart from the ITAM in the cytoplasmic domains of each CD3 chain have been implied in differential signalling activities of individual CD3 subunits. Especially, the highly conserved proline-rich sequence in CD3ε may be involved in early signalling events. ZAP-70 finally phosphorylates adaptor proteins such as LAT and SLP-76. Under steady-state conditions, basal levels of protein tyrosine kinase activity are balanced by the activity of protein tyrosine phosphatases such as CD45; however, this ratio is shifted following activation. It has been postulated that the concentration of TCR complexes and coreceptors in lipid rafts leads to the exclusion of CD45.

CD4 AND CD8

The protein tyrosine kinase Lck, critically required to phosphorylate the ITAM, is associated with the cytoplasmic domains of the CD4 or CD8 coreceptors. Binding of CD4 and CD8 to MHC class II and MHC class I molecules, respectively, brings Lck into proximity with the ITAM. Thus, CD4 and CD8 constitute essential components of a functional TCR complex and have the major function of bringing the Lck kinase close to the ITAM. Both chicken CD4 and CD8 have been initially characterized by specific mAb (Chan *et al*., 1988). These mAb analyses have demonstrated that CD4 and CD8 are expressed on mutually exclusive subsets of T lymphocytes in the periphery, while they are coexpressed on a major thymocyte population (see also Chapter 3). The ratio of $CD4^+$ to $CD8^+$ peripheral T cells has been detected for many different chicken lines and seems to be dependent on the MHC haplotype (Hala *et al*., 1991; Cheeseman *et al*., 2004).

Both CD4 and CD8 were cloned in a COS cell expression system (Tregaskes *et al*., 1995; Koskinen *et al*., 1999). Chicken CD4 is a single chain molecule with four extracellular Ig domains and a cytoplasmic domain with a conserved Lck binding site. Although the binding site to MHC has not been conserved a positively charged amino acids in the amino terminal domain may be involved in the interaction with MHC class II β chain.

Chicken CD8 is composed of two chains, CD8α and CD8β, which can form the two isoforms designated as CD8αα homodimer and CD8αβ heterodimer (Tregaskes *et al*., 1995). Both CD8 chains are composed of a single extracellular V-like Ig domain. The CD8α chain contains the conserved Lck binding site, whereas CD8β has only a short cytoplasmic domain. The CD8α gene seems to be polymorphic, especially in the putative MHC class I binding site (Luhtala *et al*., 1997a). Two mAb have been generated that recognize either the CD8α chain (CT8) or the CD8β chain (EP42), respectively (Chan *et al*., 1988; Tregaskes *et al*., 1995). As in mammals, most of the T lymphocytes bear the CD8αβ heterodimer. In young chickens, there is a distinct population of γδ T cells with CD8αα homodimers, mainly in the spleen and intestine (see also Chapter 2); however, in the intestine there is an additional population of γδ TCR^+ cells bearing the CD8αβ heterodimer cells that is not found in mammals (see also Chapter 13).

In contrast to the initial studies of CD4 and CD8 using a single chicken line, there is now evidence for the coexpression of both molecules on a large proportion of peripheral T cells in some chicken lines (Luhtala *et al*., 1997b). All of these cells carry the CD8αα homodimer. The function of this cellular subset is not known at present.

COSTIMULATORY MOLECULES

Optimal signalling through the TCR requires secondary signals through many different costimulatory and inhibitory receptors that regulate the extent, quality and duration of the T cell activation (Table 5.1). Structurally, these receptors can be divided in IgSF members and members of the tumour necrosis family (TNF). The IgSF members include the activating receptors CD28 and ICOS, whereas CTLA-4, PD1 and BTLA represent inhibitory receptors that contain cytoplasmic immunoreceptor tyrosine-based inhibition motif (ITIM; Greenwald *et al*., 2005). In the chicken, CD28, ICOS and CTLA-4 are clustered together on chromosome 7, PD1 is located on chromosome 9 and BTLA has not been found in the genome (Bernard *et al*., 2007).

CD28 and CTLA-4 (CD152) have a single V-like Ig domain typical for the CD28 family and they contain the typical B7 binding site MYPPPI in the FG loop (Young *et al*., 1994; Bernard *et al*., 2007). In addition, CTLA-4 has a GNGT motif in the g strand that is required for high affinity binding to the B7 family. The membrane proximal connecting peptide of CD28, but not of CTLA-4, lacks a typical C that is required for dimerization. In the cytoplasmic domain both CD28 and CTLA-4 harbour a PI3 kinase binding motif (CD28:YMXM; CTLA-4:YVKM) (Young *et al*., 1994; Bernard *et al*., 2007). To date no mAb or functional studies have been described for CTLA-4. Chicken CD28 is expressed on αβ T cells and a small γδ T cell subset (Young *et al*., 1994; Koskela *et al*., 1998). Antibodies crosslinking CD28 have a costimulatory effect on phorbol myristic acid activated T cells (Arstila *et al*., 1994) and certain mAb against CD28 induce T cells to proliferate and secrete interferon-γ (IFN-γ) without any costimulation (B. Kaspers, personal communication). This situation is similar to rat and human.

CD28 and CTLA-4 share identical ligands designated B7-1 (CD80) and B7-2 (CD86). Binding to these ligands, however, delivers opposing signals. Triggering CD28 leads to T cell activation and survival whereas CTLA-4 ligation inhibits T cell responses and regulates peripheral T cell tolerance (Greenwald *et al*., 2005). This complex situation is regulated by the affinity between receptors and ligands and the different expression of the receptors. CD28 is constitutively expressed on T cells and has a low affinity to B7 ligands. In contrast, CTLA-4 has a high affinity to B7 molecules, and it is rapidly upregulated by activated T cells. The situation is further complicated by the discovery of additional CD28 family members such as ICOS, PD-1 and BTLA, as well as novel B7 family members, including ICOS ligand (L), PD-L1, PDL2, B7-H3 and B7-H4. Not all

TABLE 5.1 Costimulatory Molecules

Human				Chicken	
Receptor	**Ligand**	**Function**	**Family**	**Receptor[a]**	**Ligand**
CD28	CD80, CD86	Activating	IgSF	CD28 (chr. 7)	CD80/86[b]
ICOS	ICOSL			ICOS (chr. 7)	Y08823
CTLA-4	CD80, CD86	Inhibitory		CTLA-4 (chr. 7)	CD80/86
PD1	PDL1			PD1 (chr. 9)	?
BTLA	HVEM			?	?
CD27	CD70	Activating	TNFR	?	?
CD30	CD30L			CD30 (chr. 21)	CD30L
4-1BB	4-1BBL			XM_417526 (chr. 21)	?
OX40	OX40L			?	?
HVEM	LIGHT, BTLA			?	?

[a]For receptors and ligands (L) that have already been described in publications, only the names are given, details are found in the text; if no published data are available accession numbers are given, question marks indicate that database mining has not revealed any chicken homologue. chr.: chromosome.

[b]One homologue of mammalian CD80 and CD86 has been described in the chicken.

of these receptor ligand pairs have been characterized for the chicken, but from genome analyses it is evident that ICOS, ICOSL and PD1 exist (Bernard *et al*., 2007). A homologue with equal similarity to both B7-1 and B7-2 has been described for the chicken; this homologue binds mammalian CTLA-4 and chicken CD28 with low affinity (O'Regan *et al*., 1999).

In contrast to the IgSF members of T cell costimulatory molecules, there is only limited information regarding the TNF receptor (TNFR) family members. Only homologues of mammalian CD30 and CD30 ligand have been characterized for the chicken (Table 5.1; Abdalla *et al*., 2004; Burgess *et al*., 2004). Chicken CD30 has only low homology with its mammalian counterparts and harbours four instead of three extracellular TNFR repeats (Burgess *et al*., 2004). The cytoplasmic binding motifs for signal transducing TNFR-associated factor (TRAF) 1, 2, and 3 molecules have been conserved, but not the site for TRAF6/TNFR-associated protein (TTRAP). A 22 amino acid long cytoplasmic region is highly conserved in human, mouse and chicken, however, its function is not known. Interestingly, chicken CD30 is strongly upregulated on cells that have been transformed with the Marek's disease virus (Burgess *et al*., 2004). This situation is similar to the diverse range of Hodgkin's and non-Hodgkin's lymphomas which overexpress CD30 – in fact CD30 was previously known as "Hodgkin's disease antigen".

The ligands of the TNFR family members are type II transmembrane molecules that share high similarity at the C-terminus and most of them are expressed on activated T cells. Chicken CD30 ligand (L) has been cloned by a substractive hybridization approach and shared about 33% identities with its mammalian counterparts (Abdalla *et al*., 2004). It is mainly expressed in spleen, thymus, bursa, lung and a monocyte-like cell line. Unfortunately, the interaction of CD30 with its potential CD30L and the functional consequences of this interaction have not been studied.

Chicken costimulatory and inhibitory receptor ligand pairs are clearly as diverse as those in mammals. Some of them have been characterized in detail but, so far, the vast majority has not been analysed.

T CELL LINEAGES

Three major chicken T cell lineages can be distinguished, based on their TCR usage and mAb reactivity. The TCR1 mAb recognizes all $\gamma\delta$ T cells, whereas the mAb TCR2 and TCR3 react

with αVβ1 and αVβ2 TCR, respectively (Chen *et al*., 1988; Cihak *et al*., 1988; Chen *et al*., 1989; Char *et al*., 1990; Lahti *et al*., 1991). αVβ1$^+$ T cells are most abundant, in young chickens, whereas γδ T cell frequency increases with age. As outlined above, CD4 and CD8 serve as additional markers to further divide these lineages into subsets. Additional mAb against various surface antigens such as CD5, CD6, costimulatory molecules (see Appendix 2) may either define more subsets or activation states of T cells. The ontogeny, tissue localization and functional aspects have been the subject of reviews (Bucy *et al*., 1991; Chen *et al*., 1996).

The MHC restriction and the division of cells into CD4 helper versus CD8 cytotoxic populations have not been thoroughly investigated in the chicken. However, several studies indicate that this mammalian concept holds true in the chicken. For instance, after infection of chickens with infectious bronchitis virus, MHC-restricted CD8$^+$ αβ T cells have been identified as effector cells (Collisson *et al*., 2000). Similarly, *in vitro* studies using reticuloendotheliosis virus (REV)-infected target cells have provided evidence that the cytotoxic effector cells are MHC restricted and reside in the CD8$^+$ αβ T cell population, whereas no cytotoxicity was found in the γδ T cell population (Merkle *et al*., 1992). In another study using a novel cytometry-based method, antigen-specific cytolytic activity was mainly confined to αVβ1 CD4$^+$ and CD8$^+$ cells (Wang *et al*., 2003). Finally, analyses of REV-transformed cell lines carrying Marek's disease virus genes have also provided evidence for MHC restriction (Uni *et al*., 1994).

The function of the two αβ T cell lineages has been analysed by depletion studies using the specific mAb. Both populations were found to be involved in cytotoxic T cell responses against REV (Merkle *et al*., 1992). In another model, the depletion of Vβ2 cells by the TCR3 mAb compromised the IgA production in bile and lung (Cihak *et al*., 1991); however, this effect could be the result of the low frequency of intestinal TCR3$^+$ cells rather than an intrinsic defect. These studies suggest that Vβ1 cells are required for the isotype switch to IgA (Merkle *et al*., 1997). Depletion of CD4$^+$ or CD8$^+$ cell populations indicated that CD4$^+$ cells are required for the development of autoimmune-mediated thyroiditis, while CD8$^+$ cells were only marginally involved (Cihak *et al*., 1998).

Although the exact role of the different αβ T cell subsets in these models remains unresolved, it is obvious from these studies that the subsets may be secreting discrete sets of cytokines. The basic concept of Th1 versus Th2 T cell subsets, which are distinguished by the secretion of either IFN-γ or IL-4 (interleukin-4) and IL-13, has not been confirmed for the chicken, mainly because of the inability to clone antigen-specific T cells. These cytokines have all been characterized in the recent years and tools to measure them by quantitative PCR-, bioassays- or antibody-based techniques are being developed (Staeheli *et al*., 2001; Avery *et al*., 2004). There is good evidence for a functional Th1 system, since the basic mechanisms leading to a functional Th1 response are well conserved in the chicken (Göbel *et al*., 2003). Vice versa Th2-like responses have been defined in some models in the chicken (Degen *et al*., 2005). To our knowledge, there have been no efforts to correlate Th1- or Th2-type cytokine secretion to Vβ1 or Vβ2 subsets.

Chickens belong to a group of animals that have high frequencies of γδ T cells compared with mice and human. Frequencies of γδ T cells in the chicken can reach up to 50% of peripheral lymphoid cells (Sowder *et al*., 1988; Kasahara *et al*., 1993). Chicken γδ T cell development is strictly thymic dependent, but differs dramatically in many aspects from αβ T cell development, such as the migration time in the thymus, the phenotype of γδ thymocytes and the inability of cyclosporine A to block γδ T cell development (Bucy *et al*., 1991). A significant androgen-induced expansion of γδ T cells is detectable in male chickens at 4–6 months of age (Arstila and Lassila, 1993). As in mammals the functional role of γδ T cells has not been elucidated. The broad tissue distribution of γδ T cells suggests that they have important immune surveillance functions. There are only few reports that have analysed chicken γδ T cells in infection models. Following oral administration of various *Salmonella enterica* Typhimurium strains, a strong increase of CD8$^+$ γδ T cells with activated phenotype was observed (Berndt and Methner, 2001; Berndt *et al*., 2006). γδ T cells were also found to participate in graft-versus-host reactions (Tsuji *et al*., 1996). In the chicken, it is not known if γδ T cells recognize classical peptide MHC antigens or, as in mammals, bind to alternative antigens. The inability to produce knock-out chickens has hampered studies on the functional roles of the different chicken T cell lineages.

Future studies using retroviral gene transfer in combination with the siRNA technology may help fill this gap (McGrew *et al*., 2004).

PERSPECTIVES

The chicken TCR serves as a paradigm for the non-mammalian TCR complex. Its components, structures and functions have been elucidated in some detail during the last decade. The continuous efforts to finalize the chicken genome project will add a good deal more information, such as the precise number of V(D)J elements, the exact structure of the TCR loci, the characterization of genes encoding chicken CD and cytokine homologues. This information will serve as an important basis to study the chicken T cell system in more detail. In addition to these molecular advances, refined techniques and tools to study chicken T cells in normal and diseased states need to be established. Major tools to be developed include mAb defining surface markers to study, as yet undefined, T cell subsets such as NK T cells or regulatory T cells; MHC tetramers to follow antigen-specific T cells after infections; *in vitro* culture methods to clone chicken T cells; mAb for intracellular cytokine staining; infection models for the analysis of T cell function *in vivo* and immune evasion mechanisms; methods to inactivate specific genes in T cells or T cell subsets *in vitro* and *in vivo*. Although the list of goals to be achieved is long, the chicken still provides an excellent model for T cell research, since it provides a one of the best characterized animal models with many advantages over other animal models. These include easy access to the embryo during egg development; advanced expressed sequence tags (EST) databases and availability of the chicken genome database; a relatively small body size so experimental subjects can be accommodated in biosecure high-containment facilities; finally, a wealth of well defined naturally occurring infectious diseases and, resistant and susceptible genotypes for studying host–pathogen interactions. All these factors provide excellent prospects for many exciting future discoveries in the field of chicken T cell research.

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6 STRUCTURE AND EVOLUTION OF AVIAN IMMUNOGLOBULINS

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INTRODUCTION
AVIAN IMMUNOGLOBULINS
AVIAN ANTIBODY RESPONSES
IMMUNOGLOBULIN HALF-LIFE
NATURAL ANTIBODIES
MATERNAL ANTIBODIES
THE CHICKEN EGG AS A SOURCE OF ANTIBODIES
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INTRODUCTION

Immunoglobulins (Ig) are glycoproteins that have antibody (Ab) activity and are found in the blood, lymph and vascularized tissues of all the jawed vertebrates (Marchalonis, 1977; Litman *et al.*, 1993). Their basic unit structure (Fig. 6.1) consists of four polypeptide chains, two heavy (H) and two light (L), that form the monomeric unit (H_2L_2). Each class of immunoglobulin can form a membrane-bound antigen receptor or a soluble secreted immunoglobulin (Fig. 6.1). Immunoglobulin G (IgG) consists of the basic unit but more complex molecules, such as IgM and IgA are made up from multiples of the unit, e.g. $(H_2L_2)_n$. The H and L chains are formed from domains, each of about 115 amino acids, which have highly conserved cysteine and tryptophan residues and an intra-domain disulphide bridge that confers the functionally important tertiary structure. The H and L chains are joined by inter-chain disulphide bonds. Domains at the amino-terminal are highly variable (V) and the V_H and V_L domain pairings create the antigen (Ag)-binding site which confers the Ab specificity. The Ag-binding cleft accommodates an epitope (antigenic site) of 6–9 amino acids or carbohydrates. Since pairs of H and L chains joined by disulphide bonds form the basic "monomeric" Ig unit (H_2L_2), it therefore possesses two Ag-binding sites (Fig. 6.1). Very little genetic variability is found in the other domains and these are referred to as the constant region domains (C_H or C_L). The H chains typically have 2–4 C region domains and the L chains a single C region domain. The biological properties are dependent on the C domains for membrane transportation; these include half-life and initiating secondary effector functions, such as complement fixation and opsonization. For a detailed description of Ig structure, the reader is referred to Burton (1987) and Turner and Owen (1993).

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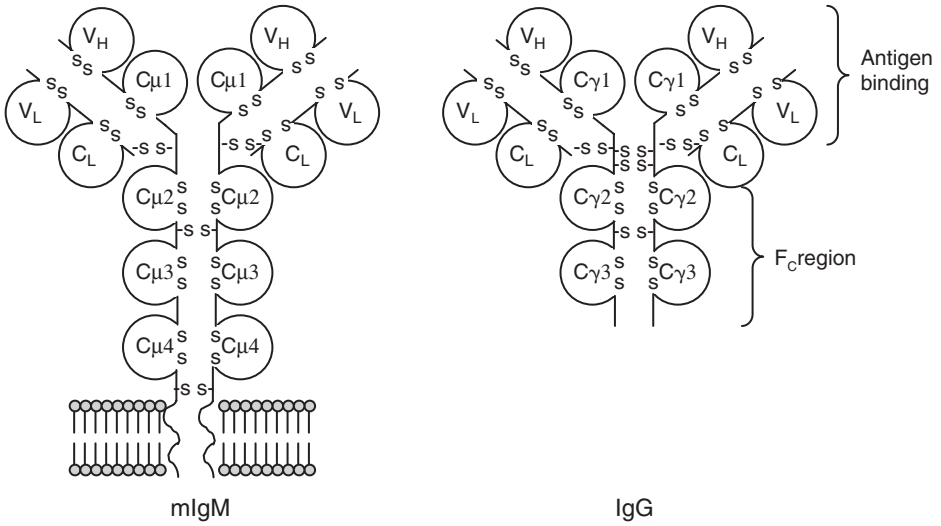

FIGURE 6.1 Schematic drawing describing the structure of the membrane-bound immunoglobulin M (mIgM) molecule and IgG. The various domains of the heavy (H) and light (L) chains are shown as variable (V) and constant (C). The C domains of the heavy chain of the IgM molecule (Cμ) and IgG molecule (Cγ) are numbered.

Advanced vertebrates possess several Ig classes, distinguished by H chains which have separately encoded C regions. These are found to be antigenically distinct when compared using antisera specific to the C_H regions of the various Ig classes. In mammals, most studies have been carried out on man and mouse and five Ig isotypes, or classes, have been identified: IgM, IgA, IgG, IgD, IgE, defined by their distinct H chains (μ, α, γ, δ and ε, respectively). These different isotypes have characteristic patterns of multimerization, for instance IgM is generally a pentamer and IgA is a dimer. Each isotype is encoded by a distinct gene and multiple heavy chain isoforms can be produced by alternative pathways of RNA processing, such as the secreted Ig and membrane Ig forms of all H chains, or full-length and truncated H chain isoforms, as has been reported for some avian species (see later). Multimeric forms of Ig contain a polypeptide joining (J) chain, which has a molecular weight (MW) of 15 kDa (McCumber and Clem, 1976). The J chain does not seem to be essential for Ig polymerization (Bouvet *et al.*, 1987), and is probably incorporated as a result of, rather than as a prelude to, Ig assembly. Evolution of the various Ig classes has probably made a significant contribution to functional diversity, as there are considerable differences in function between the human Ig classes in terms of Ag processing and recruitment of effector mechanisms. In some mammals, different subclasses of the Ig isotypes have been reported. These are encoded by separate genes but have H chains with regions of similarity; as such they may represent recent gene duplication events. In addition to isotype (class) and subclass variability, allelic variation in different individuals can give rise to different Ig allotypes.

L chains have been highly conserved in evolution (Hood *et al.*, 1967; Stanton *et al.*, 1974; Schluter *et al.*, 1990) but different types of L chains can be used. In mammals, κ and λ have been identified. Many species possess both of these isotypes, but use them in markedly different ratios (Hood *et al.*, 1967). The varying species utilization of the κ and λ isotypes is not fully understood but, since they are encoded on different chromosomes (also different from the chromosome carrying the H chain locus) and utilize different pools of V genes, there may be advantages, in terms of Ab repertoire, in using both. However, a single Ig molecule uses only one L chain isotype, just as it uses a single H chain isotype, e.g. γ_2 λ_2, and a single plasma cell normally produces Ig utilizing single H and L chain isotypes providing a single Ag specificity. The suppression of the unutilized isotypes is referred to as "allelic exclusion".

AVIAN IMMUNOGLOBULINS

Using immunocytochemical and genetic techniques, three classes of avian immunoglobulins have been identified as the homologues of mammalian IgM, IgA and IgG. Detailed reviews on their structural, physical and chemical properties, as well as the biological functions can be found elsewhere (Grey, 1969; Kubo *et al.*, 1973; Higgins, 1975; Benedict and Yamaga, 1976; Benedict and Berestecky, 1987; Higgins and Warr, 1993; Bengtén *et al.*, 2000). Although there have been detailed studies on the chicken (reviewed by Higgins, 1975) and duck (Unanue and Dixon, 1965; Grey, 1967a, b; Zimmerman *et al.*, 1971; Ng and Higgins, 1986; Magor *et al.*, 1992, 1994a, b, 1998), there has only been partial characterizations of Ig from turkey (Saif and Dohms, 1976; Goudswaard *et al.*, 1977a; Dohms *et al.*, 1978a), pigeon (Goudswaard *et al.*, 1977b), pheasant (Hersh *et al.*, 1969; Leslie and Benedict, 1969, 1970a, b), quail (Hersh *et al.*, 1969; Leslie and Benedict, 1969, 1970a, b), ostrich (Cadman *et al.*, 1994) and the numigall (a chicken/guinea fowl hybrid; Oriol *et al.*, 1972).

Avian IgM

Chicken IgM is structurally and functionally homologous to its mammalian counterpart. It is the predominant B-cell antigen receptor and during embryonic development is the first isotype to be expressed. Physical determinations on chicken IgM under various conditions have suggested a MW in the range 823–954 kDa, with an average value of 890 kDa. However, the H chain has a MW ~70 kDa and L chain 22 kDa (Leslie and Clem, 1969; Higgins, 1975) indicating that chicken IgM is more likely to have a tetrameric $(\mu_2L_2)_4$ rather than a pentameric $(\mu_2L_2)_5$ configuration; or perhaps it is a mixture of the two. Small amounts of monomeric (7S) IgM in normal chicken serum have been reported (Lebacq-Verheyden *et al.*, 1974; Higgins, 1976). Free M chains have been found in sera of bursectomized chickens (Choi and Good, 1971; Gauldie *et al.*, 1973; Higgins, 1976) and also in the survivors of infectious bursal disease virus (IBDV; (Ivanyi, 1975). The MW of duck IgM is 800 kDa and with its component H (86 kDa) and L (22 kDa) chains (Ng and Higgins, 1986), this also suggests a tetrameric structure. cDNA encoding chicken and duck μ chains have been cloned and sequenced (Dahan *et al.*, 1983; Magor *et al.*, 1998).

Invariably, IgM is the predominant isotype produced after initial exposure to a novel antigen. As in mammals, this response is usually transient (Leslie and Benedict, 1969; Higgins and Calnek, 1975a, b; Toth and Norcross, 1981; Da Silva Martins *et al.*, 1991), although after chronic bacterial infection, such as *Bordetella avium* in turkeys, IgM is reported to be active for several weeks (Suresh *et al.*, 1994). Research into the biology of avian IgM has been somewhat limited because of the apparent evolutionary conservation of the IgM molecule and its transient role in the protective immune response.

Avian IgY (IgG)

Phylogenetic studies have shown that the avian, low MW, homologue of mammalian IgG has similarities with both mammalian IgG and IgE and is probably equidistant between them. This avian isotype is the predominant form in sera, produced after IgM in the primary antibody response and the main isotype produced in the secondary response. It is often referred to in the literature as IgY, as originally proposed by Leslie and Clem (1969). Salt precipitation studies indicate that the low MW avian form of Ig has different biochemical properties to mammalian IgG, hence the name IgY. Since the chicken isotype shares homology in function, others have suggested that the term avian IgG should be retained (Ratcliffe, 2006). As a result, both IgY and IgG are used interchangeably in the literature. We prefer to use the name IgY because this Ig appears to be like the evolutionary predecessor of both IgG and IgE (see Warr *et al.*, 1995; Bengtén *et al.*, 2000), shares homology with each of these mammalian isotypes and this nomenclature is now well established for all non-mammalian vertebrates.

The major difference between the chicken IgY and the mammalian homologue is the longer H chain in the chicken molecule. Avian IgY consists of five domains (V, C1–C4), as opposed to the four that are found in mammalian IgG, and the avian molecule does not possess a genetically encoded hinge. Instead, there are "switch" regions with limited flexibility at the Cυ1–Cυ2 and the Cυ3–Cυ4 domain interfaces. The limited flexibility of the avian IgY may account for some of the unique biochemical properties, such as the inability to precipitate antigens at physiological salt concentrations, seen in chickens and ducks. For example, the two arms may be so closely aligned that they preclude cross-linking of epitopes on large antigens (reviewed in Warr *et al.*, 1995). The Cυ2 domain may have been condensed in subsequent evolution to become the genetic hinge found in mammalian IgG.

Although IgY is the major avian systemic Ab active in infections, detailed characterization has only been carried out in the chicken and in the duck. Chicken IgY in serum is monomeric, H_2L_2, with MW 165–206 kDa (Tenenhouse and Deutsch, 1966; Orlans, 1968; Leslie and Clem, 1969; Gallagher and Voss, 1969, 1970), although a 19S polymer has been detected in sera of day-old chicks (Higgins, 1976). The MW of υ chain is 67–68 kDa (Leslie and Clem, 1969; Oriol *et al.*, 1972). For detailed information on the physical, chemical and antigenic properties of chicken IgY, the reader is referred to other reviews (Kubo *et al.*, 1973; Higgins, 1975). The amino acid sequence of the υ chain and disulphide-bonding patterns has been deduced from the cDNA nucleotide sequence (Parvari *et al.*, 1988). Partial characterizations of turkey IgY (Saif and Dohms, 1976; Goudswaard *et al.*, 1977a) and pigeon IgY (Goudswaard *et al.*, 1977b) show similarities with that of the chicken. Radioimmunoassay analyses have indicated antigenic similarities between avian IgY molecules (Hädge and Ambrosius, 1986) and confirmed a lack of antigenic relationship with mammalian IgG or IgD (Hädge *et al.*, 1980a, b). A relationship with mammalian IgA (Hädge and Ambrosius, 1984a) has been reported but this is now discounted. A problem encountered with structural studies on IgY from many avian species is the unusual pattern of degradation in response to reducing agents and proteolytic enzymes. The molecules dissociate following mild reduction in the absence of a dispersing agent (Leslie and Benedict, 1969) and papain hydrolysis yields predominantly F(ab′) fragments and dialyzable peptides rather than the $F(ab')_2$ and Fc fragments obtained with IgG (Leslie and Benedict, 1970a).

As mentioned above, ducks produce two "isoforms" of IgY (Higgins and Warr, 1993; Warr *et al.*, 1995). The larger is structurally analogous to chicken IgY and has been referred to as "7.8S IgG" (Unanue and Dixon, 1965; Grey, 1967a, b; Zimmerman *et al.*, 1971). The smaller isoform ("5.7S IgG" is sometimes referred to as IgY(ΔFc)), since it has only three H chain domains (V, C1 and C2) and resembles, structurally and antigenically, an $F(ab')_2$ fragment of IgY (Grey, 1967a; Zimmerman *et al.*, 1971; Hädge and Ambrosius, 1984b; Magor *et al.*, 1992). Further details on IgY(ΔFc) can be found in Chapter 21.

The full-length and truncated υ chains (plus a third species which occurs as a transmembrane (TM), Ag-receptor form on the surface of some lymphocytes) are products of a single gene (Fig. 6.2; Magor *et al.*, 1994a). The three υ chains arise from the use of different polyadenylation cleavage sites. In the case of the υ(ΔFc) chain, the cleavage site follows a small exon ("Term") lying in the intron between the Cυ2 and Cυ3 exons. The Term exon encodes the two terminal amino acids (glutamate–phenylalanine) of the υ(ΔFc) chain, which are the only amino acids occurring in this polypeptide that do not also occur in a comparable position within the full-length υ chain.

The evolutionary origins of the truncated IgY are unknown. It is not yet known whether the truncated IgY occurs in all the waterfowl or only ducks and geese; however, it is thought to be present in all Anseriformes. A truncated IgY occurs in some species of turtles. Some other species (lungfish, nurse shark and skate) also have truncated Ig, however these are not IgY, suggesting that truncated Ig has arisen more than once during evolution (Ota *et al.*, 2003).

Avian IgA

The predominant form of Ab activity in bodily secretions is IgA. In mammals, IgA is a dimer $(\alpha_2L_2)_2$ linked by a J chain that binds to a receptor on the tissue surface of epithelial cells (Underdown and Schiff, 1986; Kerr, 1990). This receptor becomes integrated into the IgA

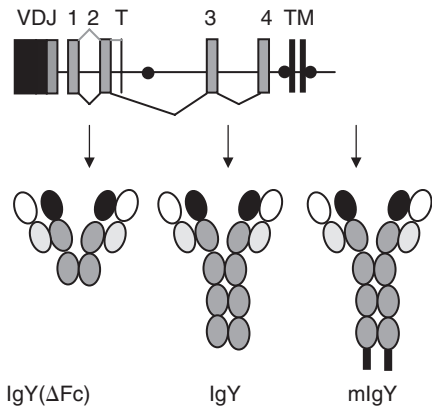

FIGURE 6.2 Schematic drawings showing how the full-length and truncated υ chains of duck IgY are the products of a single gene. Alternate splicing to generate the truncated υ chain includes the first two C exons and alternate terminal exon T as shown above the line. Splicing to generate the full-length υ chain skips the alternate internal exon as shown below the line. Splicing to generate the TM form includes the TM exons, which are spliced into a cryptic splice site near the end of exon 4. Each transcript uses a different polyadenylation signal sequence.

molecule as secretory component (SC) and the IgA complex is then transported through the epithelial cell and secreted into the lumen of the organ in question (Solari and Kraehenbuhl, 1985). SC promotes adhesion of IgA to the epithelial surface and protection from proteolytic degradation within the cells.

IgA has been found in all mammals and, based on molecular genetic evidence from chicken (Mansikka, 1992) and duck (Magor *et al.*, 1998), it is probably present in all avian species. The phylogenetic origins of IgA are not known. Hsu *et al.* (1985) have reported that the African clawed frog (*Xenopus laevis*) has a secretory molecule (IgX) that has retained more of the physical and antigenic properties of IgM and is secreted into the intestinal tract. However, it does not associate with the J chain, nor has an SC been identified in frogs. *Xenopus* IgX is considered an analogue of IgA because sequence differences suggest that it is not a homologue (Mussman *et al.*, 1996).

Several workers have demonstrated the presence of a structurally and functionally homologous form of mammalian IgA in chicken secretions, especially bile (Bienenstock *et al.*, 1972; Lebacq-Verheyden *et al.*, 1972, 1974; Orlans and Rose, 1972). cDNA encoding the chicken α chain has been sequenced, and the inferred amino acid sequence confirms overall homology with mammalian α chains (Mansikka, 1992). There are some differences, however, the chicken α chain possesses four C domains with no genetic hinge suggesting that (as with the relationship between avian and mammalian IgG) the primitive C_H2 domain could have been condensed to give the hinge region of the mammalian molecule. The molecule contains J chain (Kobayashi *et al.*, 1973) and SC (Watanabe *et al.*, 1975; Parry and Porter, 1978; Peppard *et al.*, 1983, 1986). Chicken IgA, extracted from secretions such as bile, is usually larger than the IgA found in mammalian secretions suggesting the avian form is a trimer $(\alpha_2L_2)_3$ or a tetramer $(\alpha_2L_2)_4$ (Watanabe and Kobayashi, 1974). Recent cloning of cDNA encoding chicken J chain (Takahashi *et al.*, 2000) and polymeric Ig receptor (Wieland *et al.*, 2004) confirms that the origins of secretory Ig predate the evolutionary divergence of birds and mammals.

Molecules cross-reacting antigenically with chicken IgA have been identified in guinea fowl, pheasant, Japanese quail, turkey and pigeon, but not duck (Parry and Aitken, 1975). IgA has since been confirmed biochemically and antigenically (but not yet characterized at the molecular level) in the turkey (Goudswaard *et al.*, 1977a, 1978; Dohms *et al.*, 1978a) and in the pigeon (Goudswaard *et al.*, 1977b, 1979).

A secretory form of Ig found in the bile of ducks and geese was shown to resemble IgM, both physically and antigenically (Ng and Higgins, 1986; Hädge and Ambrosius, 1988a, b).

Yet, in terms of ontogeny, this secretory form of Ig appeared in the bile several weeks after IgM had appeared in the serum (Ng and Higgins, 1986; Higgins *et al.*, 1988). Using cDNA cloning and sequencing (Magor *et al.*, 1998) has shown that the H chains resemble chicken α chains and studies on the expression of α chain mRNA have confirmed the delayed ontogeny of this molecule. A cDNA corresponding to the polymeric Ig receptor and J chain were also identified in duck (Lundqvist *et al.*, 2006).

Lack of Avian Homologues of IgD and IgE

Although there were some early reports of a chicken homologue of IgD on the surface of chicken lymphocytes (Chen *et al.*, 1982), it is generally accepted that there is no avian homologue for Igδ, with the majority of chicken B cells expressing IgM. Likewise no IgE isotype has been described for birds. Avian IgY can sensitize tissues (Celada and Ramos, 1961; Kubo and Benedict, 1968; Faith and Clem, 1973; Chand and Eyre, 1976), although the parameters of sensitization and activation differ from those of mammalian reaginic Ab (Garcia *et al.*, 1988). It seems likely that functions ascribed to IgE are performed by avian IgY.

L Chains

The L chains from a variety of avian species have been studied (Hood *et al.*, 1967; Kubo *et al.*, 1970, 1971; Grant *et al.*, 1971; Leslie, 1977; Reynaud *et al.*, 1983, 1985, 1987; McCormack *et al.*, 1989; Carlson *et al.*, 1990; Ferradini *et al.*, 1994; Magor *et al.*, 1994b). Most interest has concerned the mechanism of generation of Ab diversity (see Chapter 4) rather than the structural biochemistry. Early studies suggested chicken L chains were like the mammalian κ chain (Hood *et al.*, 1967); however, it is generally agreed that the avian L chains align with the λ L chains of mammals. Antigenic studies have been confirmed by sequence analysis of cDNA clones (Reynaud *et al.*, 1983; Magor *et al.*, 1994b). There is some physicochemical and antigenic evidence that avian species have additional L chain isotypes (Leslie, 1977; Ng and Higgins, 1986), which could reflect heterogeneity within the λ class (e.g. due to glycosylation).

Genomic Organization of the IgH Locus

With three isotype genes available, Southern blotting has been used to map the duck IgH. The picture which emerged from these Southern analyses suggested an unusual organization with the υ gene 3′ in the locus. Overlapping λ phage genomic clones were isolated and mapped to determine the gene order. The α chain gene and the υ gene were determined to be in inverse orientation (Magor *et al.*, 1999). Subsequent complete sequencing of the overlapping genomic clones showed that the duck locus was organized with α between μ and υ, and α in inverse transcriptional orientation (Fig. 6.3(a); Lundqvist *et al.*, 2001). The contig generated extended from a D segment, included a single J segment, followed by μ, α and υ heavy chain genes. The single J segment suggested that ducks, like chickens, undergo limited rearrangement events by VDJ recombination. It was also apparent that there is no avian homologue of IgD in the locus. Class switching to α involves an unusual intermediate requiring the inversion of a segment carrying μ and α rather than the deletional switch circle seen in mammals (Fig. 6.3(b)).

Despite its importance, the chicken IgH locus has received less scrutiny than that of the duck. Long-range PCR on genomic DNA confirmed a similar organization of the υ and α genes of the chicken (Zhao *et al.*, 2000). Although bacterial artificial chromosome (BAC) clones were identified that carried the υ gene, these extend almost 100 kb downstream, and do not carry the α or μ genes (Zhao *et al.*, 2000). Previous work examined the germline configuration of the chicken μ gene, and demonstrated the presence of a single J_H segment in the chicken heavy chain locus (Kitao *et al.*, 1996). At the time of writing, the chicken IgH locus has not yet been assembled from the draft of the chicken genome.

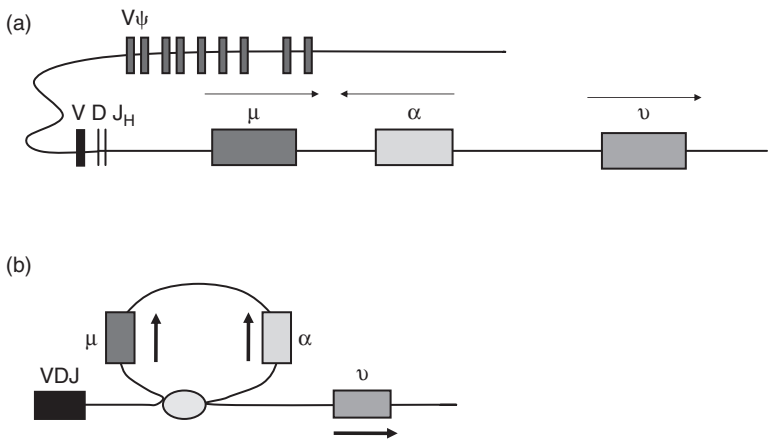

FIGURE 6.3 (a) Schematic drawing derived from overlapping genomic clones of the duck immunoglobulin (Ig) locus, showing the gene organization with α between μ and υ, and α in inverse transcriptional orientation. Upstream pseudogenes are inferred from data showing ducks undergo limited VDJ rearrangement events and are diversified by gene conversion. (b) Class switching to Ig α involves an unusual intermediate requiring the inversion of a segment carrying μ and α rather than the deletional switch circle in mammals. Class switching to υ would proceed through the excision of a switch circle.

The chicken light chain locus has recently been mapped to chromosome 15, in a region syntenic to the human L light chain locus (http://www.ensembl.org/Gallus_gallus/). The locus contains a single functional V_L and J_L region upstream of one C region. Upstream of the locus are approximately 25 V region pseudogenes, which lack the recombination signal sequences required for VDJ recombination, and have 5′ and/or 3′ truncations. The pseudogenes are used in a "hyperconversion" mechanism to generate the diversity at the light chain, involving gene conversion of fragments of the pseudogenes into the rearranged functional V region gene (Reynaud *et al.*, 1987).

The generation of the chicken Ig repertoire is reviewed in Chapter 4. Similarly, a recent review has examined duck V_H and V_L region sequences in expressed sequences. The data suggest that the same gene conversion paradigm for development of the immune repertoire holds true in ducks (Lundqvist *et al.*, 2006).

Avian Immunoglobulin Allotypes

A number of allotypic variants associated with chicken μ, α and υ chains have been reported (reviewed by Wakeland and Benedict, 1975; Ch'ng and Benedict, 1982; Benedict and Berestecky, 1987). These may have some importance in immune regulation (Ivanyi and Hudson, 1979; Ratcliffe, 1984). Allotypic forms of IgY have been reported to exist in the sera of turkey and pheasant (Ch'ng and Benedict, 1981) based on reactivity with Ab against eight allotypes associated with chicken IgY but not in other avian species examined. However, nucleotide and amino acid sequence analysis of υ cDNA has indicated the probable occurrence of allotypic variants in duck IgY (Magor *et al.*, 1992). In comparisons of cDNA sequences and genomic sequences for the duck, allotypes were observed for all genes, however there were 37 polymorphic positions in the α gene, compared with 10 and 7 in μ and υ, respectively (Lundqvist *et al.*, 2001). Many of these polymorphic positions were within the Cα2 domain suggesting this region is evolving rapidly or under less constraint.

AVIAN ANTIBODY RESPONSES

As for mammals, the IgM is the predominant isoform produced in the avian primary humoral response while IgY is the predominant form in the secondary response. Most studies on Ab

responses have used the chicken. A wide variety of antigens and assays have been employed in such studies (Kubo *et al.*, 1973), but most of the published work relates to protective immunity generated in response to pathogens or vaccines. It has long been recognized that chickens have a prodigious ability to produce antibodies in response to specified antigens (Goodman *et al.*, 1951). However, there can be marked differences in the abilities of different inbred lines to produce Ab responses to the same antigen or vaccine (for further discussion, see Chapter 8 and Fig. 8.3). Studies of Ab production and function in other avian species have been much less comprehensive. Bearing in mind that the class *Aves* is both large and diverse, it seems highly likely there will be considerable species variation, both the ability to produce Ab and in the properties of the primary and secondary Ab responses (Higgins, 1996).

Measurement of Ab responses to complex antigens such as those found in pathogens or vaccines provide little information on the intricacies of Ab responses, such as the contribution of the different Ig isotypes during the course of a primary or secondary response. The response to defined Ag, such as purified proteins or heterologous red blood cells, can be more useful for studies on Ig function, the ontogeny of the humoral immune system or as indices of the immunosuppressive effects of toxins. Single antigenic determinants or "haptens" (e.g. dinitrophenol (DNP), trinitrophenol (TNP) or fluorescein) are required for more precise analyses of Ag–Ab interactions that yield information on affinity, valency and kinetics of the response. Such approaches have been limited to the chicken with few studies carried out on other avian species.

Early biochemical studies on the precipitation of chicken IgY indicated an unusual feature. Optimum precipitation, assessed by the titre and intensity of precipitin lines, occurs in a high salt (10× physiological) or low pH (<5.0) environment (Hektoen, 1918; Banovitz and Wolfe, 1959; Benedict *et al.*, 1963; Gallagher and Voss, 1970). Precipitating Ab from pheasant, quail and owl have similar requirements, although those from duck, pigeon and turkey do not (Goodman and Wolfe, 1952; Kubo and Benedict, 1969). It is generally considered that salt and low pH support (by undefined mechanisms) the extensive cross-linking needed to generate insoluble complexes of Ag and avian Ig. Higgins (1996) reviewed the subject, eliminating most of the explanations. He concluded that the high salt concentration and low pH affects the steric arrangement of the F(ab) arms of the avian IgY and alters the shape of the molecule producing functional bivalency (see Higgins, 1996).

Analysis of the literature reveals that avian species show a broad spectrum of humoral immune responsiveness. However, it is pertinent to point out here that in direct comparisons of the Ab responses between chickens and of other species birds Ab responsiveness is in the order chicken>>pheasant/partridge>>>turkey>pigeon>quail>duck (Higgins, 1996).

Secretory IgA provides a first line of defence against many pathogens. Preferential stimulation of secretory IgA production probably requires delivery of the Ag to the mucous membranes and the use of live organisms. Secretory IgA against mycoplasmas (Barbour *et al.*, 1988; Benćina *et al.*, 1991), coccidia (Davis *et al.*, 1978; Mockett and Rose, 1986), *Escherichia coli* (Parry *et al.*, 1977) and Newcastle disease virus (NDV; Lee and Hanson, 1975) have been detected in chicken secretions. By comparison there have been few studies on IgA in other avian species. Turkeys develop IgA antibodies to *B. avium* (Suresh *et al.*, 1994) and ducks produce bile IgA Ab to influenza A viruses after experimental infection (Higgins *et al.*, 1988). In the chicken, Ab against NDV is found in respiratory secretions after intranasal, but not intramuscular, administration of vaccine (Zakay-Rones *et al.*, 1971). In the duck bile the secretory IgA response to orally administered live influenza viruses is independent of systemic Ab (Higgins *et al.*, 1988). Unfortunately, secretions enriched in IgA are not readily obtained from live birds.

The contribution made by affinity maturation to the development of specificity and effectiveness of avian Ab is not clear. Affinity maturation is one outcome of the somatic mutational events that occur in the maturing B cells during the immune response. With the generation of an increasingly diverse repertoire of Abs, some will have higher affinities for the Ag and will dominate subsequent responses. In mammals, this occurs as the Ab response switches from IgM to IgG.

A major question is whether the avian variable region genes, constructed by recombination and gene conversion, have abilities to undergo further diversification during subsequent immune responses. In chickens, Ig gene diversification in germinal centres has only been examined for the light chain, as the upstream V_L pseudogene sequences are entirely known. The mutation due to gene conversion continues in chicken splenic germinal centres, with evidence suggesting that more than one event accumulates in a single V region sequence (Arakawa *et al.*, 1996). The accumulation of gene conversion events in the V_L chain sequences appeared more targeted than seen in a similar analysis of bursal gene conversion events (Arakawa *et al.*, 1998). In addition, there was a much greater diversity of sequences following immunization with fluorescein isothiocyanate (FITC)-bovine serum albumin (BSA), a larger antigen, than DNP-BSA. While this is suggestive of affinity maturation, there is no evidence to confirm that these mutational events result in antibodies of higher affinity. Recent re-analysis of these data suggests that the diversification in chickens (and rabbits) tends towards more mutation and less selection than in mammals, reflected in the bushier tree structures (Mehr *et al.*, 2004). Somatic mutation, seen as unlinked point mutations, was apparent in chicken splenic V_L region sequences, suggesting that this mechanism of diversification is operating in chicken Ig genes (Arakawa *et al.*, 1996, 1998). It seems possible therefore, that ongoing gene conversion can contribute some additional diversity that is provided by somatic mutation in mammals. Nevertheless, the efficiency of this system, in terms of both repertoire development and increasing affinities, might be very low.

IMMUNOGLOBULIN HALF-LIFE

Turnover rates of chicken Ig have been determined by several investigators using radiolabelled proteins. Ivanyi *et al.* (1964) prepared chicken gamma globulin (chGG) using ammonium sulphate precipitation of serum proteins and labelled this protein fraction with ^{131}I. The biological half-life was determined to be 2 days in 14-week-old males and 1.55 days in adult females. Using a similar approach (Patterson *et al.*, 1962b) determined chGG half-life in newly hatched chickens and laying hens and reported values of 3 and 1.45 days, respectively. The increased turnover chGG in laying hens can be explained by the transfer of IgY from the circulation to the developing ovarian follicles (see section Maternal Antibodies). A more detailed study was carried out by Leslie and Clem (1970) using purified radiolabelled chicken IgY and IgM to measure serum elimination curves in roosters. They observed a rapid clearance phase during the first 48 h followed by a slower elimination with first-order kinetics. The initial rapid clearance was attributed to intra- and extra-vascular equilibration and the removal of aggregated or denatured proteins. Serum half-lives were extrapolated during the second phase and found to be 4.1 days for IgY and 1.7 days for IgM. In agreement with this study, Herlyn *et al.* (1977) calculated the half-life of purified non-aggregated IgY in 2–9-month-old chickens to be 3.3 ± 0.7 days. Values for turkey Ig have been provided in only one study (Dohms *et al.*, 1978b). Turkey IgY had an average half-life of 5.91 days in laying hens. The values for IgM measured in only two hens were 2.69 and 2.74 days. This study also reported the half-life of IgA for two birds as 1.92 and 1.68 days. Comparable data for IgA in two chickens were 3.3 and 1.9 days (Kaspers, 1989).

As an alternative approach, the decline of antigen-specific maternal antibody titres in newly hatched chicks has been used to study IgY elimination kinetics. Maternally derived infectious bronchitis virus-specific antibodies exhibited a linear decline with a mean half-life of 5–6 days (Darbyshire and Peters, 1985), an observation confirmed by Fahey *et al.* (1987) who reported a half-life of 6.7 days for IBDV-specific maternal antibodies. Maternal IBDV-specific antibodies were reported to decline to background levels within 28 days of hatching (Knoblich *et al.*, 2000). Similar elimination kinetics were reported for NDV-specific maternal antibodies. Using haemagglutination inhibition assays, Kaleta *et al.* (1977) estimated the half-life of maternal antibodies in chicks as 5.2 days. Injection of antibodies into maternal antibody-negative chicks led to nearly identical results, a half-life of 4.9 days. This study also demonstrated that 4-week-old chicks and adults have more rapid elimination kinetics (3.5 and 3.4 days, respectively) than the

very young chicks (Kaleta *et al.*, 1977). The faster elimination kinetics observed in antibody injection studies is compared with studies based on naturally transferred maternal antibodies may partly be due to the removal of denatured proteins in the injected preparations. Collectively, these studies give some guidance on calculating titres of antigen-specific maternal antibody at a given time point, on the basis of single quantitative analysis.

NATURAL ANTIBODIES

Natural Abs have been defined as antigen-binding Ab present in non-immunized individuals. In mammals, these antibodies are preferentially derived from the $CD5^+$ B cell population (Casali and Notkins, 1989) located in the peritoneal cavity and along the intestinal tract (Quan *et al.*, 1997; Ochsenbein *et al.*, 1999). Natural Abs have broad specificity but only low binding affinity (Dacie, 1950; Casali and Notkins, 1989). In mammals, these Abs are mostly IgM, although IgG and IgA isotypes have been reported (for review, see Quan *et al.*, 1997). Natural Abs are considered part of the innate immune system and it has been suggested that in mammals they cooperate with the complement system as a first line of defence (Thornton *et al.*, 1994). They can perform various other functions, such as being involved in the clearance of foreign, dead or catabolic materials, e.g. polysaccharides; enhancing antigen uptake, processing and presentation by B cells or dendritic cells. It has also been suggested that natural Abs contribute to self-tolerance for a considerable part of the binding repertoire of natural Ab in the human is directed against auto-antigens, such as thyroglobulin, myoglobulin, ferritin, transferrin, albumin and cytochrome C (Avrameas, 1991).

Parmentier *et al.* (2004) investigated the phenomenon of natural Ab in the plasma of chickens that had been divergently selected for high or low specific antibody responses to sheep red blood cells. Using a range of different self and foreign antigens (chicken albumen, ovalbumin, myoglobulin, thyroglobulin, transferrin, keyhole limpet haemocyanin (KLH) and BSA), these workers concluded that natural Ab are present in chickens that have not been immunized. They reported an increase in the levels of natural Ab with age, perhaps due to environmental sensitization, and higher levels in those birds that had been selected for better antibody responses to sheep red blood cells. In a further study, Parmentier *et al.* (2004) isolated natural Ab that bound to a specific antigen (KLH) from the plasma of non-immunized adult hens and adoptively transferred it to young cockerels which had not been immunized. Adoptive transfer of the natural Ab modulated a subsequent humoral immune response to KLH, whereas when specific antibodies isolated from birds that had been immunized with KLH were adoptively transferred the "naïve" recipients showed no enhanced humoral response after KLH immunization. Lammers *et al.* (2004) concluded that natural Ab could play an important role in both the initiation and the regulation of specific humoral immune responses of poultry. The functional relationship between natural Ab and specific antibodies remains unclear and it would be useful to investigate the genetic and functional relationships between these two types of Ab. The role of natural Ab in the regulation of immune responses and its importance for disease resistance, especially in the neonate chick, deserves further investigation.

MATERNAL ANTIBODIES

The immune system of newly hatched chick is only partially mature and therefore is not capable of providing complete protection against pathogens upon its first encounter with the external environment after hatching. Innate immune mechanisms seem to be fully functional in the neonate but optimal adaptive immune responses only develop during the first few weeks after hatching. The B lymphocytes first emigrate from the bursa to seed secondary lymphoid organs about 3 days before hatching, whereas the first population of T cells leaves the thymus around embryonic incubation day (EID) 6, with the second and third waves of migrations taking place about EID 12 and around the time of hatching (discussed in Chapter 3). Transfer of maternal antibodies helps to protect the offspring until adaptive immune responses become

fully effective. This was first described by Felix Klemperer (1893), who observed that in birds, immunity against tetanus bacteria is transmitted via the egg yolk. It was later addressed by Brierley and Hemmings (1956), who demonstrated the selective transport of antibodies from the yolk sac to the circulation of the embryo and hatched chick. These and other studies laid the foundations for the concept that in birds maternal antibodies are deposited into the egg and subsequently absorbed by the developing embryo (Brambell, 1970). The phenomenon has been exploited in vaccination strategies by the poultry industry; point-of-lay pullets are usually boosted with vaccines to raise their circulating antibody levels during lay, so that protective maternal antibodies are transferred to the offspring. For a fuller discussion of practical vaccination strategies, see Chapter 20.

IgY is selectively secreted from the circulation of the hen into the egg yolk (Patterson *et al.*, 1962a, b). The amount of IgY transferred across the follicular epithelium into the yolk is proportional to the IgY concentrations in serum (Kramer and Cho, 1970; Loeken and Roth, 1983; Al-Natour *et al.*, 2004; Hamal *et al.*, 2006) and mediated by an active transport mechanism (Kowalczyk *et al.*, 1985). Antigen-specific IgY antibodies are found in the newly laid egg with a delay of 5–6 days in comparison with the serum antibody concentration. This is explained by the time required for follicular development and oviposition (Patterson, 1962b). Yolk IgY concentrations were reported to be in the range of 20–25 mg/ml (Rose *et al.*, 1974), 7.9 mg/ml (Kowalczyk *et al.*, 1985) and 10 mg/ml (Lösch *et al.*, 1981) for the chicken, depending on the analytical method used. For turkey and pigeon values of 5.1 and 5.4 mg/ml, respectively, have been reported (Goudswaard *et al.*, 1978; Engberg *et al.*, 1992). The rate of accumulation of IgY in the egg is proportional to the increase in mass of the developing oocyst and estimated to be 45 mg/day at 2 days before laying (Kowalczyk *et al.*, 1985). The mechanism of this transfer is still not known but is likely to be receptor mediated (Loeken and Roth, 1983). Studies in the duck further indicate that a functional Fc portion is involved in IgY secretion into the yolk since (IgYΔFc), the naturally occurring truncated form of IgY in duck serum, is only poorly transferred to the yolk (Liu and Higgins, 1990).

The second step in maternal antibody transfer requires absorption of IgY across the yolk sac membrane into the embryonic circulation. This transport process begins at a slow rate around EID 7 (Kramer and Cho, 1970) and increases steeply during the 3 days before hatching to reach a capacity of 600 μg/day (Kowalczyk *et al.*, 1985). It has long been proposed that this process is Fc receptor-mediated since chicken IgY – but not mammalian IgG – is capable of being transported, and binding of IgY to the yolk sac membrane is pH dependent (Tressler and Roth, 1987). Recently, the receptor has been cloned and named FcRY (West *et al.*, 2004). Unexpectedly, this receptor is not homologous to the neonatal major histocompatibility complex (MHC) class I related receptor FcRn responsible for intestinal and placental absorption of IgG in mammals but belongs to the mannose receptor family (see Chapter 7).

The total amount of IgY absorbed by the embryo represents only 10% of that deposited into the egg yolk. The fate of the remaining 90% is not known, though most likely it is digested proteolytically along with the residual yolk contents (Kowalczyk *et al.*, 1985). This may explain the approximate 8-fold lower antigen-specific antibody titres in neonatal serum compared with those in yolk. Total serum IgY levels increase to their maximum value about 2 days after hatching, reaching 1–5 mg/ml (Kowalczyk *et al.*, 1985), and subsequently they decline until *de novo* synthesis of IgY becomes evident. Comparable kinetics have been described for the duck, with peak levels at day 5 and minimum levels at day 14 after hatching (Liu and Higgins, 1990).

While IgY is only found in the yolk, but not the albumen, the converse is true for IgM and IgA antibodies. Only minute amounts of these two isotypes can be detected in the yolk (Yamamoto, 1975), with most being secreted into the egg white (Rose and Orlans, 1981), reaching concentrations of 0.15 and 0.7 mg/ml for IgM and IgA, respectively (Rose *et al.*, 1974). During embryonic development these isotypes are distributed to the yolk sac and the amniotic fluid (Kaspers *et al.*, 1991) but not transferred into the embryonic circulation (Higgins, 1975). Since the amniotic fluid is imbibed by the embryo at about EID 10–12 both IgM and IgA are found in the gut at hatching. Additional Ig is delivered to the intestine when the remaining

contents of the yolk sac are transferred through the yolk stalk during the first 2 days after hatching; they probably play an important role in the early protection of the gut.

Transfer of maternal antibodies to the offspring via egg yolk is evident in all avian species studied thus far (Rose *et al.*, 1974; Dohms *et al.*, 1978b; Liu and Higgins, 1990; Engberg *et al.*, 1992). However, some species have developed additional pathways to protect their hatchlings; the best characterized example is that of the pigeon (Goudswaard *et al.*, 1979). Birds such as pigeons, penguins and greater flamingos produce a specific secretion in the crop sac which is regurgitated to feed to the squabs. Crop milk is rich in fats and proteins, although unlike mammalian milk it does not contain lactose. Moreover, the production of crop milk is under the control of prolactin (Anderson *et al.*, 1984). Crop milk is also rich in IgA with concentrations in the range of 1.5 mg/ml, though it contains little IgY. Uptake of IgA from crop milk leads to IgA accumulation in the intestinal tract where it presumably provides protection against pathogenic gut microorganisms. Very little (Engberg *et al.*, 1992), or no (Goudswaard *et al.*, 1979), IgA is transferred across the intestinal wall into the circulation and therefore this represents an important local immunity in the gut.

THE CHICKEN EGG AS A SOURCE OF ANTIBODIES

As already pointed out, immunization of hens leads to the generation of antigen-specific IgY antibodies which are efficiently transferred into the yolk (Rose and Orlans, 1981). Extraction of these antibodies from freshly laid eggs can be easily accomplished without the need for regular blood sampling or specialized equipment. Here, we will give a brief overview of the generation, purification and application of chicken egg antibodies.

Since the chicken egg yolk contains between 8 and 10 mg IgY per ml (Kowalczyk *et al.*, 1985), about 100–200 mg of total IgY can be extracted from a single egg (Lösch *et al.*, 1986) with 2–10% being antigen specific (Schade *et al.*, 1994). Specific chicken antibodies have been successfully raised against a wide variety of antigens including proteins, peptides, lipid hormones and carbohydrate components from a large variety of species including viruses, bacteria, fungi, plants and animals (Schade *et al.*, 1991). A major advantage of the chicken is that antibodies can be raised against antigens that are highly conserved in different species of mammals; this has been attributed to the phylogenetic distance between birds and mammals (Gasmann *et al.*, 1990; Larsson *et al.*, 1993). In addition, chicken IgY antibodies show little cross-reaction with mammalian Ig (Hädge and Ambrosius, 1984a), and do not bind to mammalian Fc receptors (Larsson *et al.*, 1992; Lindahl *et al.*, 1992) or activate the mammalian complement pathway (Larsson and Mellstedt, 1992). Therefore, avian antisera can be used to avoid the interference, normally associated with the use of mammalian antibodies, in various immunological assays (Larsson and Sjoquist, 1988; Zrein *et al.*, 1988), flow cytometry (Lindahl *et al.*, 1992) and immunohistology (Schmidt *et al.*, 1993). Earlier studies have reported that IgY antibodies require unique buffer systems for agar gel immunoprecipitation assays (Hersh and Benedict, 1966; see above), but subsequent work has shown that immune complex formation can be achieved under the same conditions as used for mammalian antisera (Altschuh *et al.*, 1984; Bade and Stegemann, 1984).

Chickens are most frequently immunized into the pectoral muscle with antigens in combination with adjuvants. Use of complete Freund's adjuvant (CFA) was reported by some authors to be well tolerated producing no local inflammatory reactions (Gasmann *et al.*, 1990). However, detailed histological studies have clearly documented that CFA elicits all signs of inflammation and leads to the development of a persistent granulomatous myositis (Wanke *et al.*, 1996). Despite attempts to develop alternative adjuvants with comparable immunogenicity but less inflammatory potential, CFA still remains the standard adjuvant in many laboratories. New technologies such as DNA vaccination may well overcome some of the problems associated with immunization with conventional antigens and facilitate the generation of yolk antibodies against specific antigens (Cova, 2005). As in rabbits, the initial immunization with soluble antigens is usually followed by a booster immunization 2–3 weeks later. In most cases, a specific high titre IgY response develops 1–2 weeks later and collection of eggs for IgY extraction

from yolks should be started 2–3 weeks after the booster injection. Subsequent booster immunizations may help to maintain the antibody titre over long periods of time (Orlans, 1967; Rose *et al.*, 1974), but rarely leads to a significant increase in titre.

Several methods have been described for large-scale antibody purification from the egg yolk. In the first stage it is essential to remove the lipids, either by precipitation without (Akita and Nakai, 1992) or with chemical additives (Jensenius *et al.*, 1981; Polson *et al.*, 1985) or using lipid solvents such as ether (Patterson *et al.*, 1962a, b), chloroform (Aulisio and Shelokov, 1967) or isopropanol (Bade and Stegemann, 1984). Usually, this is followed by one or more protein precipitation steps (Jensenius *et al.*, 1981; Polson *et al.*, 1985) or the use of chromatographic methods.

Avian Antibodies as Tools for Research

Antibodies extracted from egg yolk have found widespread application in biomedical research (Tini *et al.*, 2002). Certain proteins are so highly conserved between different mammalian species that it is impossible to evoke xenogenic responses. In contrast, birds have sufficient evolutionary distance from mammals for the mammalian proteins to be recognized as foreign and avian humoral immune responses to be evoked. For instance, it has proved extremely difficult to produce mammalian antibodies against the abnormal form of the prion protein (PrP), whereas antibodies could be raised in chickens and quantities harvested from eggs for use as diagnostic tools for studies on bovine spongiform encephalopathy (Somerville *et al.*, 1997).

Finally, there has been little emphasis on the development of avian monoclonal antibodies. A chicken B cell line (HU3R27), deficient in thymidine kinase activity, was developed as a fusion partner for the production of chicken monoclonal antibodies but the resulting hybridomas soon lost the ability to produce antibody in culture (Nishinaka *et al.*, 1989). An improved fusion cell line (R27H4) was subsequently developed by fusing HU3R27 with spleen cells from a chicken immunized with KLH and the resulting hybridoma reported to secrete highly reactive IgY and weakly reactive IgM for over 6 months (Nishinaka *et al.*, 1991). Since then a small number of other chicken monoclonal antibodies have been produced using either R27H4 or the related R27H1 cell line as fusion partners (Asaoka *et al.*, 1992; Lillehoj *et al.*, 1994; Nishinaka *et al.*, 1996; Constantinoiu *et al.*, 2003), although this technology seems to have found only limited use. Perhaps this is because avian antibodies are only required as tools for relatively few specialized assay systems and sufficient amounts of these purified Ab can normally be provided from egg yolk. Moreover, the well developed, and robust, mouse monoclonal antibody technology can generally supply most of the tools necessary for investigating avian immune responses.

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7 AVIAN INNATE IMMUNE RESPONSES

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INTRODUCTION
CONSTITUTIVE BARRIERS
ACTIVE RESPONSES (CHEMICAL)
ANTIMICROBIAL PEPTIDES
THE COMPLEMENT SYSTEM
CELLS OF THE INNATE IMMUNE SYSTEM
PATTERN RECOGNITION RECEPTORS
SUMMARY
REFERENCES

INTRODUCTION

Traditionally, the innate immune system was viewed as a scavenger system that fights invading pathogens immediately after they enter the body. However, it is now clear that there is a complex interplay between innate and adaptive responses. For many years, phagocytosis and lysis defined innate immune responses but this view has changed to include first line of defence, pattern recognition and immune modulation. Initial encounter of pathogens with the innate system leads not only to the destruction of the pathogens but, in many cases, initiates a cascade of events, including recruitment of various immune components, as well as induction and modulation of the adaptive immune system. In this chapter, we will review components of the avian innate immune system with emphasis on those elements not dealt with elsewhere in the book. For instance, detailed summaries of cells such as macrophages and dendritic cells (DC) are the subject of Chapter 9. Nonetheless, it is important to first provide a generalized description of innate immune components. As with many other aspects of the avian immunology, most research has been carried out with the chicken and relatively little is known about the innate systems of other avian species.

Innate immunity is expressed in a variety of guises, from the initial response to physical and chemical attack by invading microorganisms to the co-ordinated recruitment and action of a series of specialized cell populations. Broadly speaking, there are innate mechanisms that operate as a constitutive barrier function, including the nature of epithelial surfaces and the molecules that coat them, such as mucus and antimicrobial chemical components (e.g. lysozyme).

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There are also inducible biochemical responses, including alterations in mucus composition and increased secretion of constitutive components as well as production of antimicrobial compounds (e.g. defensins). The complement system can also be considered as an inducible cascade that liberates both antimicrobial elements (e.g. the C9 membrane attack complex (MAC)) and elements that attract and modulate the cellular immune system. The cellular components of the innate immune system range from the action of specialized epithelial cells to the activity of more classically defined immune cell populations, such as macrophages, granulocytes (polymorphonuclear cells), thrombocytes and natural killer (NK) cell populations.

Classically, innate responses are considered important in the earliest phases of microbial invasion, limiting the spread of the pathogen until adaptive responses (B and T cell mediated) become mobilized to clear the infection. In this sense, one should consider why innate responses are more rapid than adaptive responses; the nature of the pathogen recognition elements are fundamental to this distinction. With the innate system, pathogen recognition is mediated by elements encoded in the germline DNA. This contrasts with the receptors on B and T cells (BCR and TCR, respectively) which are formed by a tightly regulated rearrangement of receptor gene elements, a process which provides an immense diversity of subtly different receptors. As a consequence, innate receptors have less diverse specificity for invading microorganisms but are more abundant throughout the body than individual TCR or BCR. For example, in the cellular innate immune system up to ~100 different receptors are expressed at a relatively high frequency, with particular distributions and bias according to the cell type. Hence, each receptor is usually expressed on millions of innate immune cells (and often also expressed on adaptive immune cells). In contrast, an individual TCR or BCR is characteristic of a very small population of cells derived from a specific clone which was successfully selected during B or T cell development (see Chapters 4 and 5). With T cells the number of clones in the naïve (unexposed) population has been estimated in the region of 10–100 cells in an overall pool of about 100 million. The different responses of innate and adaptive immunity are a consequence of the diversity and frequency of receptors used to sense the invading pathogens. Where the frequency of the pathogen detection receptor is high, the response can be very rapid but the ability to differentiate between pathogens (the specificity) is low. With diverse clonally expressed BCR and TCR, the rate of the response generated is slow (dependent on cell proliferation) but the specificity of the response is very high.

Another characteristic that differentiates innate from adaptive responses is immunological memory, while the latter responds more efficiently to second exposure the magnitude of the innate response is not influenced by pre-exposure and simply reflects the immediate stimulus (e.g. numbers of microorganisms). The capacity of adaptive cell populations to respond faster to second exposure is chiefly achieved in two ways: by altering the activation requirements for specific (previously activated) clones of cells and by increasing the frequency of the specific (memory) cells in the population, thereby increasing the frequency of extremely rare receptors. In contrast, the frequency of receptor expression in innate cell populations is already high and the retention of a proportion of previously exposed cells does not alter the specificity of the repertoire for pathogen recognition. Hence, the genetic conformation of the pathogen recognition receptors – germline or rearranged – and their role in development of cell populations (e.g. B and T cells are entirely dependent on signalling through the BCR and TCR) are probably the most important differences between the responses we define as innate and adaptive. However, exposure to a particular stimulus can alter the numbers of NK cells expressing a particular distribution of receptors thereby introducing bias in the NK cell pool and affording a degree of increased responsiveness to recently encountered pathogens (Yokoyama and Kim, 2006). This feature of the response will change the rate of NK cell responsiveness to a previously encountered pathogen and could be regarded as increased short term preparedness for rechallenge.

Although often considered separately, as in this book, the innate and adaptive responses are highly integrated. Innate immune systems have been described in many organisms, including invertebrates that do not possess an adaptive immune system, and in such cases function in

isolation. However, with higher vertebrates, which use specialized rearrangement processes to generate the adaptive cell populations, it is difficult to separate functionally the *in vivo* activity of innate and adaptive responses. Indeed, the earliest pathogen recognition events that occur in the body lead to recruitment and enhancement of innate responses, as well as activation of the adaptive immune system. The local innate response at the site of infection also serves to recruit cells of the developing adaptive (and innate) immune response by virtue of chemotactic molecules (e.g. some complement components and chemokines) and alteration of receptors on endothelial cells lining the vessels.

Innate activation of a variety of cell subsets can increase their capacity to interact with T cells of the adaptive system, the most important of these are the DC subsets. These cells are reviewed in Chapter 9 but we should point out here that the DC play a unique integrating role in the immune system – these being the only cells that can activate naïve T cell subsets by virtue of their high levels of major histocompatibility complex (MHC) molecules and co-stimulatory activity (e.g. expressing CD80/86). The DC not only activate naïve T cells but also direct the type of T cell response which develops using a variety of mechanisms including secretion of cytokines such as interleukin (IL)-10, IL-12 and IL-18 (see Chapter 10 for a full review of cytokines and chemokines). Immature DC reside in the tissues where they sample their environment; exposure to particular pathogen components leads to DC activation and migration to areas where they can contact naïve T cells (typically lymph nodes in mammals and equivalent structures in birds reviewed in Chapter 2). During the migration phase DC mature, manifest as a reduction in phagocytic capacity, increased expression of MHC molecules and surface co-stimulatory molecules. Depending on the spectrum of pathogen molecules activating the DC (by interaction with pattern recognition receptors (PRR) discussed below), DC mature differently, providing the capacity to differentially influence downstream T cell responses.

The innate immune system is not only involved in initiating and directing adaptive responses but is also often used as an effector arm of the adaptive response; hence, it is often inappropriate to delineate innate- and adaptive-mediated killing of invading pathogens in all but the very initial phases of the infection. Examples of such interactions are many and varied, here we will highlight a few to illustrate the importance of "innate cell types" in expression of adaptive immunity. Of these interactions, perhaps, the easiest to comprehend is that between the T cell and macrophage. The macrophage is firmly placed within the spectrum of innate cell types and performs a variety of functions, including phagocytosis and production of antimicrobial compounds such as the reactive oxygen intermediates and nitric oxide (NO) (see Chapter 9). However, ongoing T cell responses often involve the production of interferon gamma (IFNγ), a cytokine that has the ability to activate macrophages, increasing their capacity to phagocytose and kill invading microorganisms. In this circumstance, the macrophage can be viewed as one of the effector arms of the T cell response. Secondly, a range of phagocytic cells, including macrophages and polymorphonuclear cells, express Fc receptors that bind immunoglobulin (produced by B cells) to enhance recognition and engulfment of foreign pathogen-derived material. In such cases, the phagocytic cells of the innate system act within the context of antibody- and B cell-mediated immunity. A third example of the integration of adaptive and innate responses includes the influence of T cells on specialized epithelial cell subsets such as goblet cells (mucus producing), paneth cells and intermediate cells in the gut; altering their numbers, inducing secretion of products (e.g. defensins) and altering the chemical composition of mucus.

In this introduction, we have explored the organization of the innate immune system in terms of structural, chemical and cellular components, some of which are expressed constitutively, while others are inducible. We have also compared and contrasted receptor-based constraints on the innate and adaptive immune systems to offer a clear discrimination between these systems. We have highlighted the complexity of the interactions between the innate and adaptive immune systems to illustrate the level of intercommunication and integration that underpins immunity *in vivo*. This notwithstanding, it is impractical to deliver the details within different areas of innate immunity without separating these into comprehensible segments. In the following

sections, we cover the main areas of innate immunity and refer the reader to other chapters for details on macrophages (Chapter 9), cytokines and chemokines (Chapter 10) that will supplement and support. As with all other areas of avian immunology the recent availability of the *Gallus gallus* genome sequence (Hillier *et al*., 2004) has allowed identification of the repertoire of conserved immune components which will, over the next few years, revolutionize our capacity to study and understand the innate immune mechanisms that birds use to combat all classes of invading pathogens.

CONSTITUTIVE BARRIERS

Intact physical barriers are the first essential line of defence to protect the host against pathogen invasion. This is best illustrated after their breakdown, which can result in lesions in the skin, the airways and other mucosal surfaces with increased risk of infection. Cannibalism, observed in all kinds of rearing systems, is a very obvious prelude to infections. Ectoparasites such as the red dust mite or infections with helminths also breakdown the natural barriers. Although not of host origin, the normal flora present on body surfaces also help to prevent colonization by pathogens. This is the basis for prophylaxis designated as "competitive exclusion" (Van Immerseel *et al*., 2005) which is much used by the poultry industry. For instance, to prevent salmonella infections, day-old chicks are treated with undefined mixtures of normal gastrointestinal flora to enhance col-onization with harmless commensal flora and compete with pathogens.

Other mechanisms such as ciliary movement in the airways, fatty acids on the skin, peristaltic movement of the intestine, the gastric acidic pH, secretion of mucus and antimicrobial peptides (AMP) cumulatively serve as highly effective barriers to infection. Apart from the AMP, discussed below, other mechanisms have not been given detailed study. Additionally, there are many descriptions of immune evasion strategies which have been evolved to circumvent the host's physical, chemical and microbial defence mechanisms.

ACTIVE RESPONSES (CHEMICAL)

Acute Phase Proteins

Inflammation in vertebrates is accompanied by a large number of systemic and metabolic changes, collectively referred to as the acute phase response. Stimuli which commonly give rise to acute phase responses include bacterial infection, surgical or other trauma, bone fractures, neoplasms, burn injuries, tissue infractions and various immunologically mediated inflammatory states. Among the changes in homeostatic settings described during the acute phase response are fever, somnolence, anorexia, increased synthesis of a number of endocrine hormones, decreased erythropoiesis, thrombocytopenia, alteration in plasma cation concentration, inhibition of bone formation, negative nitrogen balance with consequent gluconeogenesis, alterations in lipid metabolism and, finally, major alterations in the concentration of some plasma proteins called the acute phase proteins (APP).

APP have empirically been defined as those proteins whose plasma concentrations change by 25% or more following an inflammatory stimulus. Proteins which increase during the response are designated positive APP, while those that decline are termed negative APP. Furthermore, the positive APP can be divided into three groups: those increasing by 50% (group I), those increasing 2- to 5-fold (group II), and those increasing up to a 1000-fold (group III).

Some APP increase in concentration as early as 4 h after the inflammatory stimulus, attain a maximum level within 24–72 h but decline very quickly. Other APP begin to increase 24–48 h after stimulus, reach a maximum in about 7–10 days returning to normal after about 2 weeks.

The biological functions of APP can be divided into three major types: participation in host adaptation or defence, inhibition of serine proteinases and transport of proteins with antioxidant activity.

Host Defence Acute Phase Proteins

Members of this group are: C-reactive protein (CRP), mannan-binding lectin (MBL) and fibrinogen (FB). A major function of CRP and MBL is to bind to foreign pathogens and damaged host cells to initiate their elimination by interaction with phagocytic cells or activation of the complement system. FB plays a major role in homeostasis, tissue repair and wound healing. FB and fibrin interact with endothelial cells, promoting the adhesion, mobility and cytoskeletal organization of these cells.

Inhibitors of Serine Proteinases (Serpins)

Serine proteinases (serpins) control extracellular matrix turnover, fribrinolysis and complement activation. Since inflammation leads to the activation of a number of serine proteinases and the release of others from phagocytic cells, serpins play a critical role in limiting the activity of these enzymes, thus protecting the integrity of host tissues. To this group belongs the α1-proteinase inhibitor, which inhibits neutrophil elastase, α1-antichymotrypsin, which inhibits chrymotrypsin-like serine proteinases, and C1 inhibitor, which inactivates the blood coagulation factors XIIa and XIIf.

Transport Proteins with Antioxidant Activity

APP belonging to this group play an important role in protecting host tissues from toxic oxygen metabolites released from phagocytic cells during the inflammatory state. The APP caeruloplasmin is involved in copper transport and antioxidant defence, haptoglobulin binds haemoglobulin released during haemolysis, and haemopexin binds haeme released from damaged haeme-containing proteins.

Most positive APP are glycoproteins synthesized in the liver upon stimulation with pro-inflammatory cytokines and glucocorticoids and subsequently released into the blood stream. The main pro-inflammatory cytokines involved are IL-6, IL-1, and tumour necrosis factor (TNF)-α. These cytokines are produced by tissue macrophages and peripheral blood monocytes, the most likely cells to initiate the acute phase response as they become activated upon encounter with foreign organisms. Over a period of 12–24 h the liver responds to the cytokine exposure and starts to produce the positive APP and decrease the production of the negative APP. Extrahepatic production of APP has been reported in most mammalian species studied. Finally, psychological stress has been shown to elevate the level of IL-6 and APP. It is not known how stress induces the acute phase response, but activation of the hypothalamic–pituitary–adrenal axis may trigger systemic or local cytokine production by stress signals, thereby stimulating the production of APP (Murata *et al*., 2004).

C-Reactive Protein

CRP plays an important role in the protection against infection, clearance of damaged tissue, prevention of autoimmunity and regulation of the inflammatory response. It has both pro- and anti-inflammatory effects *in vitro* and *in vivo*. The blood CRP level increases by varying amounts in response to a variety of bacteria and intracellular antigens of damaged cells, followed by opsonization by phagocytic cells or by activation of the complement system (Murata *et al*., 2004). In chickens, natural infection with the protozoan parasites such as *Eimeria* spp. and *Histomonas* induce high levels of CRP (Chamanza *et al*., 1999).

Serum Amyloid A

Serum amyloid A is an acute phase apolipoprotein of the high-density lipoprotein fraction of plasma. Its role in the defence during inflammation is not well understood but various effects have been reported. In chickens, it is a major APP increasing 10- to 100-fold upon stimulation with *Escherichia coli* or *Staphylococcus aureus*. Chicken serum amyloid A is known to be the precursor of amyloid A protein. Individuals with chronically increased serum amyloid A may develop amyloidosis characterized by fibrillar deposition of amyloid A in internal organs such as liver, spleen and joints. In the poultry industry there is an increasing interest in this protein since amyloid arthropathy is a major problem on layer farms (Chamanza *et al.*, 1999).

α1-Acid Glycoprotein

α1-acid glycoprotein is a natural anti-inflammatory agent that inhibits neutrophil activation and increases the secretion of an IL-1 receptor antagonist by macrophages. It may also be involved in the clearance of lipopolysaccharide (LPS) by direct binding thus neutralizing its toxicity (Murata *et al.*, 2004). In chickens, α1-acid glycoprotein is a major APP increasing 10- to 100-fold upon stimulation. High levels have been recorded during infections with infectious bronchitis, infectious laryngotracheitis, infectious bursal disease viruses, *E. coli*, and *Salmonella enterica* serovar Enteritidis (Chamanza *et al.*, 1999).

Haptoglobulin

Haptoglobulin binds free haemoglobulin, which is toxic and pro-inflammatory, in plasma and reduces the oxidative damage associated due to haemolysis (Murata *et al.*, 2004). Chicken haptoglobulin is composed of three molecular variants that react differentially with the lectin concanavalin A (Delers *et al.*, 1988). Although chicken haptoglobulin has been isolated, changes in levels in common chicken diseases have not been investigated.

Haemopexin

The role of haemopexin is to bind and transport free haeme to the liver where it is internalized and degraded, thus preventing haeme-mediated oxidative stress and haeme-bound iron loss (Stred *et al.*, 2003). Many bacteria require iron for rapid growth but iron sequestered to haemopexin cannot be used by most bacteria so inhibiting their growth. After treatment with *E. coli* LPS chicken haemopexin increased 3-fold starting after 6–12 h, reaching a peak within 24 h but returning to the basal level by 14 days (Adler *et al.*, 2001).

Fibrinogen

FB is involved in homeostasis, providing a substrate for fibrin formation, and in tissue repair providing a matrix for the migration of inflammatory-related cells (Murata *et al.*, 2004). After treatment with turpentine, chicken FB was found to rise 3- to 4-fold, peak after 3 days and return to the basal level after 7 days (Amrani *et al.*, 1986).

Fibronectin

Fibronectin influences a wide range of cellular properties such as growth, differentiation, migration and apoptosis (Ruoslahti and Reed, 1994) and interacts with macromolecules, cells and bacteria (Mosher and Furcht, 1981). After treating chickens with turpentine or LPS plasma fibronectin concentration increases 3- to 4-fold, peaking after 48 h and returning to basal levels after 7 days. Chicken hepatocytes secrete increased levels of fibronectin after treatment with glucocorticoid or chicken IL-6 (Chamanza *et al.*, 1999).

Caeruloplasmin

Caeruloplasmin is a copper-containing ferroxidase which oxidises toxic iron to its non-toxic ferric form. Caeruloplasmin can act as an anti-inflammatory agent reducing the number of neutrophils that attach to the endothelium, and as an extracellular scavenger of peroxide (Murata *et al*., 2004). In chickens, caeruloplasmin is a moderate APP increasing 10- to 100-fold upon stimulation. Injection with *E. coli* endotoxin immediately produces a 50% increase in chicken plasma caeruloplasmin and higher levels have been reported after infection with *Eimeria tenella* (Chamanza *et al*., 1999).

Transferrin–Ovotransferrin

Transferrin may be involved in sequestering ferric ions to prevent pathogens and parasites from utilizing them (Murata *et al*., 2004). It is a negative APP in mammals but a positive one in chickens. Treatment with turpentine, LPS injection or reticuloendothelial virus infection increases chicken transferrin within 1–3 days. It has recently been shown that chicken ovotransferrin is actually the same molecule as transferrin (Xie *et al*., 2002c). It is a moderate APP increasing 10- to 100-fold upon inflammatory stimulation. Elevated serum IL-6 concentrations and increased numbers of heterophils preceded an increase in ovotransferrin (Xie *et al*., 2002b), which peaked after 3 days, remained high at 5 days and returned to a basal levels after 10 days (Xie *et al*., 2002c). There is some evidence that ovotransferrin can modulate macrophage and heterophil function in chickens (Xie *et al*., 2002a).

Collagenous Lectins

Collagenous lectins are multimeric proteins, such as the collectins and ficolins. The collectin family are calcium-dependent multimeric proteins with carbohydrate-binding domains (CRD) aligned in a manner that facilitates binding to microbial surface polysaccharides or host macromolecules and contribute to their removal. Eight different collectins have so far been identified, including MBL, surfactant protein A (SP-A), surfactant protein D (SP-D), collectin liver 1 (CL-L1), collectin placenta 1 (CL-P1) and conglutinin (CG), collectin of 43 kDa (CL-43) and collectin of 46 kDa (CL-46). By contrast, the ficolin family does not bind in a calcium-dependent way; three different ficolins have been identified in humans (Holmskov *et al*., 2003).

The basic subunit of the collectins is a trimer with three identical monomers each containing four regions: a cysteine-rich N-terminal domain, a collagen-like region, a coiled-coil neck domain and finally a large globular CRD (Fig. 7.1).

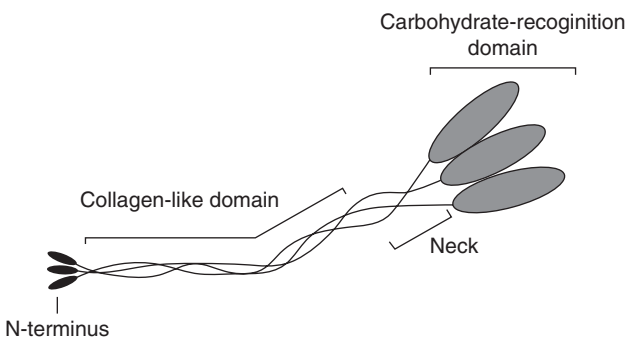

FIGURE 7.1 Domain organization of the collagenous lectin trimer. Trimers are comprised of monomers containing a cysteine-rich N-terminal domain, a collagen-like domain, a neck region and a C-terminal carbohydrate-recognition domain (with permission from Lillie *et al*., 2005).

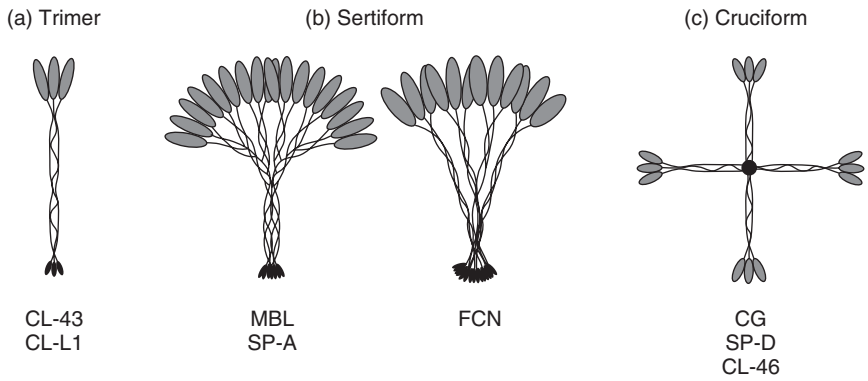

FIGURE 7.2 The multimeric structures of collagenous lectins in animals. (a) CL-L1 and CL-43 have a trimeric native form. (b) MBL, SP-A, and FCN form sertiform (bundle of tulips) oligomers comprised of varying numbers of trimers. (c) CG, SP-D, and CL-46 form cruciform oligomers comprised of four trimers. CL-P1 exists as membrane-bound trimers (not shown) (with permission from Lillie *et al.*, 2005).

The three polypeptide chains forming the trimer also assemble into larger oligomers via cysteine residues in the N-terminal domain except for CL-43 and CL-L1 for which the trimer is the native form (Holmskov *et al.*, 2003). MBL, SP-A and ficolin may consist of up to six subunits forming a "bouquet of tulips". By contrast, the collectins CG, SP-D and CL-46 are only assembled with four subunits in a cruciform structure (Holmskov *et al.*, 1995, 2003; Fig. 7.2).

The binding affinity of a single lectin domain to carbohydrates is very low. However, three lectin domains held together as a single subunit have a higher avidity to carbohydrate-rich surfaces as shown for CL-43 (Holmskov *et al.*, 1995). The avidity is even higher in those collagenous lectins that create higher-order multimers. Depolymerization is therefore expected to lead to the loss of binding affinity and biological function for carbohydrate-rich surfaces (Holmskov *et al.*, 1994).

A number of potential receptors for the collectins on phagocytic cells have been identified. The first to be described was C1qR. In addition, the receptor for C3b, CR1 has been shown to interact with C1q and MBL, but none of these potential receptors are definitive and so will not be discussed further.

Mannan-Binding Lectin

MBL is primarily produced in the liver and secreted into the blood, although, low expression has also been demonstrated in the lung, thymus, kidney, small intestine and testis (Wagner *et al.*, 2003). MBL increases up to 3-fold after an infection, which is why MBL is defined as an APP (Thiel *et al.*, 1992).

Chicken serum lectin has been isolated by affinity chromatography (Laursen *et al.*, 1995; Laursen and Nielsen, 2000). Although two polypeptides (molecular weight (MW) 33 and 34 kDa) were isolated, NH2-terminal sequencing revealed identical sequences over the first 30 amino acid residues, indicating the existence of only one MBL form in chickens (chMBL). It was shown, using electron microscopy, that the chMBL is an oligomer of subunits, each composed of three 30–40 kDa polypeptides joined in a collagen-like triple helix with three globular CRD (Laursen *et al.*, 1995).

Measurements of the level and distribution of MBL in different tissues during embryogenesis and through early and adult life (Laursen *et al.*, 1998a; Nielsen *et al.*, 1998) show that serum MBL increased from 11 days before hatching until 1 year of age, although levels varied between different types of birds. The level in egg yolk was comparable to that in serum but none was found in the egg white (Laursen *et al.*, 1998a). It is considered likely that maternal

chMBL, like maternal immunoglobulin Y (IgY), is transported from the yolk sac to the embryo and, after hatching, chMBL is catabolized whilst endogenous MBL is synthesized in the chick (Laursen *et al*., 1998a).

Nielsen *et al*. (1998) have investigated the level distribution and function of chMBL in different tissues after infection with infectious laryngotracheitis virus and infectious bursal disease virus. In non-infected chickens, chMBL was found in the cytoplasm of a few liver cells but increased levels were found in the liver from virus-infected chickens suggesting an increased synthesis during acute infection. chMBL was detected on cells in germinal centres of caecal tonsils of non-infected cells, on the surface and within infectious laryngotracheitis-infected tracheal cells and in the cytoplasm of splenic macrophage-like cells from infectious bursal disease virus-infected birds. This strongly suggests that chMBL has an active role in the chicken immune system.

Chicken MBL, like the other MBL, is a weak acute phase reactant increasing only 1.2- to 2-fold in serum after infection (Juul-Madsen *et al*., 2003). Complement activation is directly associated with the concentration of MBL in serum (Juul-Madsen *et al*., 2003) supporting the hypothesis that the level of serum MBL affects the degree of virus neutralization before the adaptive immune response takes over. MBL could influence the level of the specific antibody response or direct the immune response towards a more cellular response.

Ficolin

Ficolins were originally discovered as transforming growth factor (TGF)-β binding proteins in the porcine uterus (Ichijo *et al*., 1991), but have since been identified in many other vertebrates and invertebrates (reviewed in Matsushita and Fujita, 2001). Human L- and H-ficolin are serum proteins mainly produced in the liver (Akaiwa *et al*., 1999; Matsushita *et al*., 2000; Matsushita and Fujita, 2001). H-ficolin is also produced in the lung (Akaiwa *et al*., 1999). M-ficolin is a non-serum ficolin that is mainly synthesized by monocytes and detected on their surface, but not in the more differentiated macrophages and DC (Teh *et al*., 2000). In chickens, only one single ficolin gene has been identified, which appears to represent an undiversified ancestor of L-FCN and M-FCN (Lynch *et al*., 2005). No chemical or functional studies have been carried out thus far.

Surfactants SP-A and SP-D

The lung collectins SP-A and SP-D are mainly produced by non-ciliated bronchial epithelial cells and secreted to the lung mucosa (Crouch *et al*., 1992), but both forms have also been detected in other non-pulmonary tissues (Madsen *et al*., 2000; Akiyama *et al*., 2002). Furthermore, they have been detected at low concentrations in amniotic fluid (Miyamura *et al*., 1994). No chicken surfactant proteins have so far been isolated but the gene for SP-A has recently been sequenced (AF411083).

Other Chicken Collectins

Three chicken collectins were found and designated chicken collectins: chCL-1, chCL-2 and chCL-3 resembling the mammalian proteins CL-L1, collectin 11 and CL-P1, respectively (Hogenkamp *et al*., 2006).

ANTIMICROBIAL PEPTIDES

AMP are important components of the natural defences and have been isolated from most living organisms. Generally, they act by forming pores in the membrane of bacteria and fungi leading to cell death (Kagan *et al*., 1990). In view of the increasing problems with resistance of bacteria and fungi to commonly used antibiotics and the pressing need to find alternatives, AMP or their synthetic analogues have potential as novel pharmaceutical agents. Besides

antibacterial and anti-fungal activities some of the AMP also possess antiviral or anticancer properties and can influence inflammation, proliferation, wound healing, release of cytokines, redox homeostasis and chemotaxis (Bals, 2000).

In chickens, two classes of AMP have been identified, cathelicidin-like proteins and defensins. Recently, three cathelicidins (fowlicidin-1 to -3) have been cloned. Phylogenetic analysis revealed that fowlicidins and a group of distantly related mammalian cathelicidins known as neutrophilic granule proteins are likely to have originated from a common ancestral gene (Xiao *et al*., 2006). Fowlicidin-1 and -2 have been shown to have cytotoxic activity and binding capacity to LPS resulting in complete blockage of LPS-mediated proinflammatory gene expression. Another chicken cathelicidin CMAP27 has been cloned and shown to have marked similarities with MAP members from cattle (van Dijk *et al*., 2005).

Only β-defensins appear to exist in chickens and these may constitute the oldest form of the three defensin sub-families (α-, β-, θ-defensins; Xiao *et al*., 2004). Until now 13–14 different β-defensins, designated as gallinacins (Gal)-1 to -13, have been reported in the chicken genome clustered on the same chromosome (Sugiarto and Yu, 2004; Xiao *et al*., 2004).

Avian β-defensins may have a very important role in avian heterophils because these cells lack oxidative mechanisms (Harmon, 1998). Avian AMP have been shown to be active against a number of microorganisms. Gal-1 and Gal-1α kill *Staphyloccocus aureas, E. coli, Candida albicans, S.* Enteritidis, and *Campylobacter jejuni* but not *Pasteurella multocida* or infectious bronchitis virus (Harwig *et al*., 1994; Evans *et al*., 1995). Gal-11 was found to be predominantly active against *S.* Typhimurium and *Listeria monocytogenes* (Higgs *et al*., 2005).

THE COMPLEMENT SYSTEM

The serum complement system – a chief component of the innate immunity – is an important part of the system of defence against invasion of pathogenic microorganisms in mammals (Carroll, 2004). The complement system was initially recognized as the heat-sensitive factor in serum that "complemented" heat-stable antibody in lysis of bacteria and red blood cells. Nowadays it refers to a series of about 25 serum proteins, as well as 10 or more cell surface complement receptors and regulatory proteins that are present on a wide range of host cells. The proteins circulate in an inactive form but, in response to the recognition of molecular components of microorganisms, they become sequentially activated, working in a cascade in which the binding of one protein promotes the binding of the next protein in the cascade (see Fig. 7.3). Complement activity includes an enhancing effect on phagocytosis (opsonin activity), the ability to induce an inflammatory response, enhancing B and T cell responses (Carroll, 2004) and, finally, enhancing the direct killing of target cells (cytolysis). Complement proteins are mainly synthesized constitutively by hepatocytes and released into circulation, but macrophages are also able to synthesize the early components C1, C2, C4 and C3 (Carroll, 2004). Some factors are upregulated upon stimulation by pro-inflammatory cytokines and therefore act as APP (C3, MBL and CRP). The complement factor C3 is a key component in the system. The activation of C3 (by a proteolytic cleavage) initiates most of the biological functions of the complement system. Complement activation has been much analysed in mammals and is achieved in at least three different ways (Fig. 7.3). The classical pathway (CP) is activated by antigen–antibody complexes; the lectin pathway (LP) is activated by the interaction of microbial carbohydrates with e.g. MBL in serum and tissue fluids and the alternative pathway is activated by C3b binding to microbial surfaces and antibody molecules. These pathways differ in the manner in which they are activated but ultimately they produce the key enzyme, C3 convertase (Fig. 7.3).

Classical Pathway

The CP is primarily activated by antigen-bound IgM or IgG released after a humoral response or by natural IgM antibodies encoded by rearranged antibody genes that have not undergone

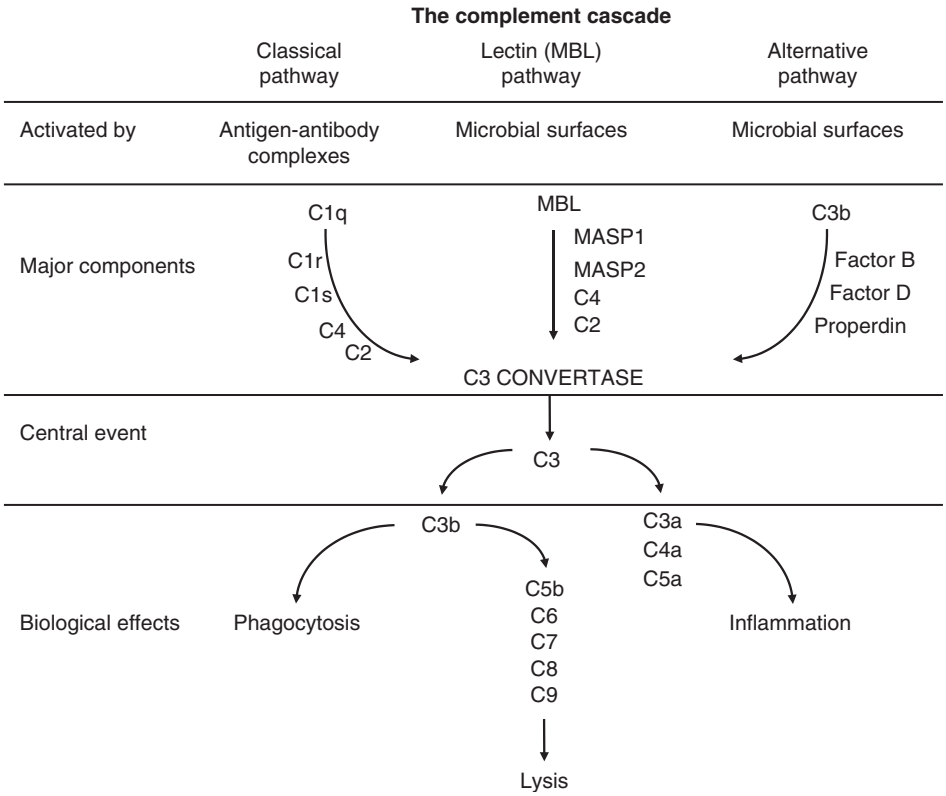

FIGURE 7.3 Overview of the main components and effector actions of the complement cascade.

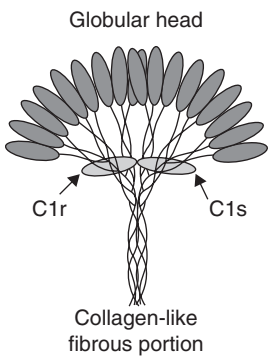

FIGURE 7.4 The first protein in the CP of complement activation C1, which is a complex of C1q, C1r, and C1s.

somatic mutation (see Chapter 6). Natural antibodies are produced by B1 cells and seem able to recognize polysaccharide antigens of microbial and viral origin (Carroll, 2004). First, C1q binds to the Fc part of the antigen-bound IgG or IgM after which the two serine proteases C1r and C1s attach to form the C1 molecule in a calcium-dependent way (Fig.7.4). C1 is the first enzyme of the pathway able to cleave and activate the next two components C2 and C4. C1q can, however, also bind directly to the surface of pathogens (certain viruses, Gram-negative bacteria) and polyanions in an antibody-independent way (Morley, 2000) and thus trigger the complement activation in the absence of antibody. C1q is a calcium-dependent sugar-binding protein, a lectin, belonging to the collectin family of proteins.

The chicken C1q molecule has been identified and has – like its human counterpart – a characteristic "bouquet of tulips" structure with six globular subunits (see above). Chicken C1q has the ability to generate full C1 haemolytic activity when mixed with the human complement subcomponents C1r and C1s (Yonemasu and Sasaki, 1986). A predicted sequence for the three chicken C1q chains was recently identified on the same chromosome (XM 417654, XM 425756, XM 417653). In addition, the sequence for chicken C1s has been determined and mapped, whereas only a "like-sequence" for chicken C1r has been found (BX 427559).

Recently, it was shown that C1q is able to bind to the APP CRP. CRP binds phosphocholine-ligands, such as modified low-density lipids, present on different bacteria and apoptotic cells, in a calcium-dependent way followed by the calcium-independent binding of CRP to C1q (Agrawal, 2005). However, this activation of the complement is not complete. The process generates C3 convertase but it is not able to generate effective C5 convertase. Therefore, it fails to assemble the MAC and may only be involved in opsonization. The C1 molecule activates C2 and C4 by cutting a small peptide (C2a and C4a) from each via the serine protease C1s (the earlier nomenclature of the small fragment of C2 was C2b and the large fragment was C2a). C4b then binds covalently to the pathogen surface and associates with C2b which has protease activity in a magnesium-dependent way. This C4bC2b molecule is the C3 convertase of CP.

No biochemical structure of C2 and C4 molecules have so far been reported for the chicken. In earlier studies, it was assumed that the role of C2 was fulfilled by the chicken factor B (Kjalke *et al*., 1993). However, the predicted amino acid sequence for C2 and the partial sequence for C4 were recently identified (XM 417722, AL 023516). In addition, C4 activity has been detected in chicken serum using human and guinea pig reagents indicating the presence of a functional C4 component in chicken; also a chicken serum protein has been found to cross-react with anti-human C4 serum (Wathen *et al*., 1987). It therefore seems reasonable to assume that chickens possess a normal classical complement pathway.

Lectin Pathway

The LP represents a recently described pathway which acts in an antibody-independent way. It is activated after recognition and binding of pathogen-associated molecular patterns (PAMP) by lectin proteins which bear CRD. To date, three members of the LP have been identified: MBL, ficolin H and ficolin L (Fujita *et al*., 2004). These molecules are equivalent to C1q in the classical complement pathway. MBL, an APP, binds to mannose or *N*-acetyl glucosamine residues present on the surface of bacteria, virus yeast and fungus. Ficolins are – like MBL – lectin-type recognition proteins also recognizing *N*-acetyl glucosamine and mannose structures (Matsushita and Fujita, 2002).

Activation of LP begins when MBL or ficolins binds to carbohydrate groups on the surface of pathogens in a calcium-dependent way and associate with the two serine proteases named MBL-associated serine proteases 1 and 2 (MASP; Lynch *et al*., 2005). The role of MASP 1 in complement activation is still debated. The MASP 1 and MASP 2 proteins are equivalent to C1r and C1s of the CP. Other MASP have also been characterized in humans namely MASP 3 and Map19, but their functions have not been fully elucidated. MASP 3 is generated from alternative splicing of the MASP 1/3 gene (Dahl *et al*., 2001). Map 19 is a truncated product of the MASP 2 gene. The lectin–MASP complex then cleaves C4 and C2 to form the C3 convertase (C4bC2a; Holmskov *et al*., 2003; Lynch *et al*., 2005).

As already discussed, chicken MBL has been isolated, mapped, cloned and characterized, in addition to the cloning of chicken MASP 2, 3 and Map 19 (NM 213587, AY 567828, AY 567830) (Lynch *et al*., 2005). Chicken MASP 1 seems to be absent, possibly due to the loss of an exon in the gene. Despite the lack of MASP 1, chicken MBL has the ability to activate complement when tested in a heterologous system by deposition of human C4 on the chicken/MASP complex. Complement activation was directly associated with the concentration of MBL in serum in a calcium-dependent way (Juul-Madsen *et al*., 2003). A single chicken ficolin gene has also been cloned and appears to represent an undiversified ancestor of the genes coding for human M-ficolin and L-ficolin (NW 060632) (Lynch *et al*., 2005). It is concluded that the chicken has a functional LP.

Alternative Pathway

In mammals a variety of "natural" activators of the alternative pathway have been characterized such as bacteria (polysaccharides, LPS), yeast (zymosan) or plants (inulin), fungi, viruses, certain mammalian cells and aggregates from immunoglobulins (Lachmann and Hughes-Jones, 1984). Activation of the alternative complement pathway begins when C3b or C3i binds to the cell wall and other surface components of microbes. C3i can be formed by hydrolysis of C3. The alternative pathway protein factor B then combines with the cell-bound C3b to form C3bB in a magnesium-dependent manner. Factor D splits the bound factor B into Bb and Ba, forming C3bBb. Finally, a serum protein called properdin stabilizes the complex by binding to the Bb to form C3bBbP which functions as a C3 convertase.

Chicken factor B is a 95 kDa glycoprotein exhibiting a genetic polymorphism with three common phenotypes (Kjalke *et al*., 1993; Koch, 1986). It is not known whether the polymorph-ism is due to single nucleotide polymorphisms (SNP) or post-translational modifications. When activated in a magnesium-dependent way, but not in a calcium-dependent way, the chicken factor B is cleaved into a Ba fragment of 37 kDa and a Bb fragment of 60 kDa leading to creation of the active C3 convertase (Jensen and Koch, 1991; Kjalke *et al*., 1993). Depletion of factor B abolished lytic activity indicating that factor B participates in the alternative pathway (Jensen and Koch, 1991). Unlike in the human genome this gene is not linked to the chicken MHC complex (Koch, 1986).

Although factor B is the only alternative pathway protein that has been characterized so far, all things considered, the results indicate that a factor B-dependent alternative pathway is active in the chicken.

C3 the Key Complement Component

When C3 convertase converts inactive C3 molecules two active components, C3a and C3b, are produced, with C3b binding covalently to the surface of pathogens, cells or other surfaces. One C4bC2b molecule is able to cleave up to one thousand C3 molecules producing many C3b molecules that can coat the pathogens' surface. Amplification of complement activation can then be achieved by association of surface-bound C3b and pro-enzyme factor B yielding the short-lived C3 convertase C3bBb of the alternative complement pathway.

Chicken C3 has been isolated, mapped and characterized (Laursen and Koch, 1989; Mavroidis *et al*., 1995). It has a double-chain structure with an α-chain (111 kDa) and a β-chain (70 kDa); both have been cloned (NM 205405). Normally, chicken C3 is present in serum, but recently it was also found in egg yolk (Recheis *et al*., 2005). Upon cleavage of chicken C3 by C3 convertase, two fragments are generated, the small anaphylatoxin C3a (15 kDa) and the major fragment C3b (175 kDa). In contrast to humans, chicken C3 exists in three molecular forms (Laursen and Koch, 1989; Mavroidis *et al*., 1995).

Complement Opsonization and Phagocytosis

In humans several proteins are able to bind and regulate complement fragments. The C4b-binding protein and factor H belong to the soluble forms of complement regulators whereas CR1 (CD35), CR2 (CD21), CR3 (CD11b/CD18), CR4 (CD11c/CD18), C1qR (calreticulin), decay-accelerating factor (CD55) and membrane cofactor protein (CD46) belong to the membrane-associated forms (Liszewski *et al*., 1991). In chickens, a cDNA encoding a complement regulatory membrane protein was recently identified. This protein showed similarities with the human decay-accelerating factor and the membrane cofactor protein (Inoue *et al*., 2001). This protein has been shown to protect mammalian transfectants from chicken complement-mediated cytolysis indicating protection of host cells from homologous C attack. In addition, predicted sequences for chicken factor H and two decay-acceleration factors have been identified and mapped (XM 426613, XM 425826, XM 417981).

Membrane Attack Complex

The MAC exists in many vertebrate species, including birds, amphibians, fish; in such species serum lyses xenogeneic erythrocytes due to complement-like reactions. When C3b molecules associate with C4bC2b complexes from the CP and LP, or with C3bBb from the alternative pathway, C5 convertase is generated. Activated C5 convertase then cleaves C5 into C5a and C5b, the final enzymatic step in the complement cascade. In mammals, the MAC involves the non-covalent association of C5b with the four terminal components C6–C9 forming a multi-molecular complex structure which is inserted into, and penetrates, the cell membrane. All these serum proteins are hydrophilic and form an amphipathic membrane-inserted pore where ions and small molecules can pass, bringing about osmotic lysis of foreign cells.

Activation of the chicken complement results in lysis when the target cells are erythrocytes, both when complement is activated in calcium-dependent and -independent ways (Ohta *et al*., 1984). There are no data on the molecular basis of this lytic reaction for avian species. However, predicted amino acid sequences have recently been determined for chickens C5–C9 suggesting a MAC process similar to that in mammalian species (XM 415405, XM 429140, XM 424775, XM 415563, AI 980217).

Anaphylatoxins

The three small split products C3a, C4a and C5a are called anaphylatoxins because they are able to perform inflammatory activities. All three proteins induce smooth muscle contraction and increase vascular permeability, but only C3a and C5a are important pro-inflammatory molecules involved in the stimulation and chemotaxis of myeloid cells bearing specific anaphylatoxin receptors (C3aR, C5aR or CD88; Gasque, 2004). The end result of the complement activation is to trigger inflammation, chemotactically attract phagocytes to the infection site, promote opsonization, cause lysis of Gram-negative bacteria and cells, expressing foreign epitopes, participate in B-cell activation and remove harmful immune complexes from the body.

CELLS OF THE INNATE IMMUNE SYSTEM

Cells of the immune system include cells of myeloid origin such as granulocytes and thrombocytes as well as NK cells that are derived from lymphoid stem cells. In mammals, various subsets of DC are considered to be either from lymphoid or myeloid progenitors. Macrophages and DC play a central role as antigen-presenting cell (APC) in immune responses and their importance is so great that they are discussed separately in Chapter 9. NK cell receptors are described in Chapter 8. Here we only provide an overview of NK cells and heterophils with special emphasis on their PRR.

NK cells

Phenotype of Chicken NK Cells

The role of chicken NK cells has been extensively reviewed elsewhere (Göbel *et al*., 1996). Here, we briefly describe the general features of NK cells and mainly focus on recent developments. NK cells represent a third lymphoid lineage which shares many features with cytotoxic T lymphocytes. Morphologically, chicken NK cells have been characterized as large lymphocytes with electron dense granula (Göbel *et al*., 1994). In contrast to development of B and T cells, NK cell development is thymus-independent (Bucy *et al*., 1989).

Just as for mammals, no unique NK cell marker has been identified for the chicken. Consequently, chicken NK cells are arbitrarily defined by their expression of the CD8$\alpha\alpha$ homodimer together with the lack of surface Ig or CD3 (Göbel *et al*., 2001). Chicken NK cells do express the common leukocyte antigen CD45 and Fc receptor-like Ig-binding activity; they

lack MHC class II antigens (Göbel *et al*., 1994). Like human foetal NK cells, chicken NK cells express cytoplasmic CD3 proteins, probably indicative of a common origin with T cells (Bucy *et al*., 1990; Göbel *et al*., 1994). More specific molecules that are solely expressed on NK cells are not currently available. The chicken genome assembly should provide information about genes that are more specific for NK cell. Potential candidates include CD122, the marker for NK cell precursors, and CD2 and CD56, antigens expressed on mature NK cells. At present, there is no information about so-called NK T cells, a mammalian subset displaying a canonical TCR in combination with NK cell markers.

Using the phenotypic criteria outlined above, Göbel and co-workers have investigated the presence of putative NK cells in a variety of different tissues such as peripheral blood, spleen, caecal tonsils, bursa, thymus and intestinal epithelial lymphocytes (IEL). Only about 0.5% of $CD3^{-}Ig^{-}CD8\alpha\alpha^{+}$ cells were detected in all tissues, except intestinal epithelium. The IEL population, however, contains a large population of putative NK cells. A monoclonal antibody (mAb) designated 28-4 has been raised against this population. IEL expressing the 28-4 antigen showed spontaneous cytotoxicitiy against the standard NK target (LSCC-RP9) cells, whereas $CD3^{+}$ intestinal T cells did not show this cytolytic activity (Göbel *et al*., 2001). The nature of the antigen recognized by the 28-4 mAb is currently unknown. Another NK cell population can be identified in the embryonic spleen around day 14. This is a time before mature T cells have seeded the peripheral tissues. The embryonic population has a similar phenotype, expresses the 28-4 antigen and has cytolytic activity (Göbel *et al*., 1994, 2001). Phenotypic studies have shown that NK cells in adult chickens reside mainly in the intestinal epithelium, with only a low frequency detected in spleen and blood. This is markedly different from man and mouse, where up to 15% of the lymphoid cells in these tissues represent NK cells.

NK Cell Function

Functional studies have detected NK cell-mediated cytotoxicity in spleen and blood cell populations. These functional analyses do not necessarily conflict with the phenotypical analyses described above, since a low frequency of NK cells could be responsible for the observed cytolytic activity. Moreover, certain infections can cause a drastic, but transient, increase in the NK cell frequency. It has been reported, that a *Mycoplasma gallisepticum* infection leads to an accumulation of TCR^{-} $CD8^{+}$ cells in the tracheal mucosa during the first week of infection (Gaunson *et al*., 2006). Sustained NK cell activity was also observed after infection with Marek's disease virus (MDV), particularly in genetically resistant chicken lines (Garcia-Camacho *et al*., 2003).

Cytotoxicity assays are normally performed with effector cells isolated from either spleen or peripheral blood obtained from either normal or infected chickens. The LSCC-RP9 target cell line was derived from a lymphoid tumour induced by the retrovirus, Rous-associated virus 2 (Sharma and Okazaki, 1981). In contrast to standard mammalian target cell lines, RP9 is MHC class I positive and even at high effector to target ratios, only a fraction of the RP9 cells is susceptible to lysis. Most other chicken cell lines, including all MDV-derived cell lines so far tested, are resistant to NK cell lysis – the same applies to mammalian-derived NK cell targets. However, it has been reported that chicken skin fibroblasts are highly sensitive for NK cell lysis (Vizler *et al*., 2002).

Culture experiments using embryonic NK cells have indicated the necessity of including growth factors to induce NK cell proliferation. Two major NK cell growth factors, IL-2 and IL-15 have been cloned (Sundick and Gill Dixon, 1997; Staeheli *et al*., 2001). Recombinant chicken IL-2 has been successfully employed to generate polyclonal NK cell *in vitro* cultures for several weeks. The effects of IL-15 have not been tested, since it has not been possible to obtain sufficient amounts of recombinant chicken IL-15 (T.W. Göbel, unpublished results).

Although many chicken cytokines have been cloned and characterized recently, their effects on NK cells are not known. Oral administration of IFNα decreased NK cell activity after MDV infection (Jarosinski *et al*., 2001), whereas *in vitro* IFNγ treatment increased NK cell activity under different circumstance (Merlino and Marsh, 2002). In conclusion, studies on NK cell function are still lacking, mainly due to the dearth of appropriate phenotypic and molecular markers.

Potential NK Cell Receptor Families

In recent years three different models of target recognition by mammalian NK cells have been described. The "missing-self recognition" postulates, that NK cells are inhibited by self proteins expressed on normal cells but which are downregulated after infection or transformation (Ljunggren and Karre, 1990). Recognition of infected cells can also be mediated by pathogen-encoded molecules. Finally, pathogens or transformation can induce the upregulation of self proteins as stated in the "induced self recognition" model. All three models have been confirmed experimentally but are not mutually exclusive. In fact, each can be mediated by the same types of receptors.

Receptors on NK cells have been grouped into two major classes that either belong to the Ig superfamily or are C-type lectins. In both these families two types of receptors can be distinguished, based on their cytoplasmic motifs and the nature of the transmembrane region. Inhibitory receptors are characterized by long cytoplasmic domains that harbour one or multiple immunoreceptor tyrosine-based inhibition motifs (ITIM). Upon phosphorylation these motifs recruit tyrosine phosphatase which prevents further downstream signalling. Activation receptors, however, have a short cytoplasmic tail and a positively charged transmembrane residue which allows their association with adapter molecules that carry cytoplasmic immunoreceptor tyrosine-based activation motifs (ITAM); this leads to activation. In many cases, activation and inhibitory receptors share a high degree of homology in their extracellular regions and may even bind similar ligands.

In mammalian NK cells there are at least two distinct receptor families: the leukocyte receptor complex (LRC) on human chromosome 19 and the NK complex (NKC) on human chromosome 12. The LRC encodes several families of Ig-like receptors such as the killer-inhibitory receptors, the Ig-like transcripts and the SIGLECs, whereas the NKC harbours multiple C-type lectin receptors including CD94 and Ly49. In the chicken, a syntenic region corresponding to the LRC represents the so-called chicken Ig-like receptor (CHIR) locus (Viertlboeck *et al.*, 2005, 2007). Other Ig-like families with multiple inhibitory and activating receptors have been located on various chicken chromo-somes and include the TREM, SIRP, CD200 and CD300 loci. Their involvement in NK cell function requires investigation.

The putative chicken NKC seems to contain only two C-type lectin molecules which have not been further characterized. Moreover, in the vicinity of the chicken MHC there are two C-type lectins designated B-Lec and B-NK. RT-PCR analyses of B-NK have indicated that it is expressed in chicken NK cells expanded *in vitro* by IL-2. It seems to be an inhibitory molecule, based on the fact that it recruits intracellular tyrosine phosphatases. The nature of its ligand is currently unknown. More details on B-NK and B-Lec are provided in the Chapter 8.

Heterophils

The counterparts of mammalian neutrophils are the avian heterophils. Like neutrophils, heterophils form the first line of cellular defence against invading microbial pathogens. They have phagocytic capability but, in contrast to mammalian neutrophils, they lack myeloperoxidase, they do not produce significant amounts of bactericidal activity by oxidative burst and their granule components seem to differ from those in mammalian neutrophils (Penniall and Spitznagel, 1975; Montali, 1988). Inflammatory stimuli such as LPS, turpentine or various infectious conditions like *E. coli* airsacculitis, staphylococcal tenosyvitis cause a dramatic influx of heterophils (Harmon, 1998). The activation of heterophils by pathogens or by cytokines induces the expression of various pro-inflammatory cytokines such as IL-1, IL-6 and IL-8 (Kogut *et al.*, 2005a, 2006). Heterophils are dependent on the expression of various PRR that are described in detail below.

PATTERN RECOGNITION RECEPTORS

The term PRR was first coined by Janeway and colleagues (Medzhitov and Janeway, 1997, 2000) to describe families of receptors that interact with conserved PAMP. These families

include soluble components (e.g. LPS-binding protein, collectins, pentraxins and alternative pathway complement components) and cell-associated components (including C-type lectins, scavenger receptors, formyl-peptide receptors, Toll-like receptors (TLR) and various intracellular receptors). Collectively, PRR families form the basis for innate immunity and underpin the ability of the host to recognize and react to invading pathogens. Many PRR are highly conserved with representatives found in diverse animal species, they provide the cornerstones upon which the antimicrobial immune response is built. Pattern recognition can be considered at three levels: interactions involving soluble PRR, those occurring at the surface of cells or within membrane-bound vesicles and, finally, those that operate within the cytoplasm. The positioning of PRR in each of these three locations covers those niches occupied by representatives of all major pathogen groups. Redundancy in recognition of particular PAMP structures and coordination of different PRR to sense any single invading class of microorganism are important features of the PRR network. In essence, the outcome of PRR-mediated responses is to initiate downstream antimicrobial cascades and cellular activities that represent the earliest events in a developing immune response. Indeed, without the activity of PRR, under most circumstances the body and the immune system will ignore the presence of non-self antigens. Moreover, the "adjuvant effect" which is central to successful vaccination, is largely mediated by PRR stimulation with microbial PAMP or their synthetic mimics.

Within the field of chicken immunology there has been a recent explosion in the study of one of these PRR families, the TLR. Before considering the role of the chicken TLR family, it is appropriate to mention the non-TLR pathogen recognition systems. These have already been described in some detail and include soluble PRR, such as LPS-binding protein, and related molecules, such as bacteriocidal/permeability-increasing protein and the "PLUNC" (palate, lung and nasal epithelial clone) molecules each of which are considered to interact with bacterial LPS. Little work on these has been undertaken using the chicken, but examples of this PRR group have been identified in the chicken genome. Other important soluble PRR include initiating components of the complement alternative pathway, such as MBL and factor B, and other members of the collectin family that has already been described.

Cell-Associated Pattern Recognition Receptors

The cell-associated PRR can be divided into those that sense PAMP at the cell surface or have been taken into intracellular membrane-bound vesicles, and those that act as cytoplasmic pathogen detection systems. The former group of PRR includes the membrane anchored C-type lectins, scavenger receptors, formyl-peptide receptors and the TLR. The membrane anchored C-type lectins include selectins, the DC-specific marker ICAM-3 grabbing non-integrin (DC-SIGN), the dectin molecules and the mannose receptor. Selectins are mostly involved in mediating cell–cell adhesion and DC-SIGN is important as an adhesion mediating T cell–DC interactions with ICAM-3 (Geijtenbeek *et al*., 2000a) and DC–endothelial interactions via ICAM-2 (Geijtenbeek *et al*., 2000b). The cytoplasmic PRR detect products of pathogens that replicate or are translocated into the cell cytoplasm (typically derived from viruses and some bacteria) and include the nucleotide-binding oligomerization domain (NOD) proteins, protein kinase receptor (PKR), retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA-5). Unfortunately, there is little published work on new TLR in birds and subsequent sections will focus on the avian TLR.

The Avian TLR

Background

The Toll protein was originally described as a regulatory protein involved in embryonic dorso-ventral axis formation of *Drosophila melanogaster* (Hashimoto *et al*., 1988) but later identified as a receptor initiating upregulation of the anti-fungal peptide, drosomycin (Lemaitre *et al*., 1996). The discovery of human TLR (Medzhitov and Janeway, 1997; Brockus *et al*., 1998;

Chaudhary *et al*., 1998) was soon followed by characterization of 13 mammalian TLR and since then members of the family have been described in fish, amphibians, reptiles and birds. A substantial body of evidence has accumulated to identify the adapter molecules, signalling pathways and downstream events. A wide range of PAMP are reported to stimulate TLR, although these are often referred to as TLR agonists because strong evidence for direct interaction of PAMP with TLR remains elusive. The most intensely studied TLR4–LPS interaction has shown that the response is dependent on, and enhanced by, complex interactions between a wide range of molecules (see below). The nature of TLR–agonist interaction is under intense scrutiny and very recently the first convincing evidence for direct interaction of TLR3 with double-stranded RNA(dsRNA) has emerged (Bell *et al*., 2006). Where extensive analysis has been undertaken it is clear that each species retains approximately 10–12 different TLR molecules and a core group of the TLR have distinct orthologues in a wide range of vertebrate species (Roach *et al*., 2005).

The general structure of TLR comprises an extracellular N-terminal leucine-rich repeat (LRR) domain with one or two cysteine-rich regions, a transmembrane domain and a C-terminal domain referred to as the Toll/IL-1 receptor (TIR) domain, reflecting a similarity with the IL-1R intracellular domain. The number and distribution of leucine-rich repeats within the ectodomain is a defining feature of specific TLR and is involved in agonist recognition. The TIR domain is relatively conserved between different TLR and contains three highly conserved regions that are important in recruitment of adapter proteins and for signalling (Akira and Takeda, 2004).

The Avian TLR Repertoire

The TLR molecules can be subdivided into phylogenetically related families and most vertebrate genomes have representatives of at least one member from each family (Roach *et al*., 2005). Based on phylogenetic analyses the main groups of vertebrate TLR can be considered ancient and are predicted to have diverged during, or before, the Cambrian period (505–590 million years ago; Roach *et al*., 2005). These families are as follows: the TLR1/2 group (with TLR1, 2, 6, 10 and *Fugu* TLR14); the TLR3 group; the TLR4 group; the TLR5 group and the TLR7 group (including TLR7, 8 and 9). Other TLR have also been described in mouse (TLR11, 12 and 13), man (a TLR11 pseudogene) and fish (e.g. the related TLR21 and TLR22 molecules). The chicken TLR (chTLR) repertoire includes representatives of the major groups and relevant components of conserved TLR signalling pathways. The first chTLR to be identified and characterized were two genes with high homology to mammalian TLR2 (Boyd *et al*., 2001; Fukui *et al*., 2001), followed by chTLR4 (Leveque *et al*., 2003), chTLR5 (Iqbal *et al*., 2005b) and chTLR7 (Philbin *et al*., 2005). Expressed sequence tags (EST) have been identified with homology to human TLR1, 6 or 10, TLR2,3,4,5 and TLR7 (Lynn *et al*., 2003; Philbin, 2006). Subsequently, identification of chTLR from the draft genome sequence (with EST annotation) has been reported (Yilmaz *et al*., 2005). Unfortunately, in this report the annotation of some chTLR sequences fails to identify the appropriate signal sequence. Two further chTLR have been identified, that are not orthologues of any mammalian TLR, and these have been termed chTLR15 and chTLR21 (Roach *et al*., 2005; Higgs *et al*., 2006; Philbin, 2006), although it is not clear that the chTLR21 is a true orthologue of fish TLR21 (see below). Despite extensive analysis no orthologues of mammalian TLR9, TLR11, TLR12 or TLR13 can be identified in any of the available sequence resources. Moreover, it is clear that remnants of a chTLR8 molecule can be detected in the chicken genomic sequence (validated by independent sequencing of this region) but this is disrupted in galliform birds with multiple frame shifts and a ~6 kB insertion containing the CR-1 element (Philbin *et al*., 2005). In summary, the chTLR repertoire to date includes at least one TLR1/6/10-like molecule, two TLR2, TLR3, TLR4, TLR5, TLR7 orthologues and two TLR that are only found in birds. Those chTLR with mammalian orthologues can be differentiated relatively easy according to amino acid identity, predicted patterns of LRR and position of the genes in areas of conserved synteny with mammalian genomes. A range of conserved components of the known TLR signalling pathways (reviewed in Akira

and Takeda, 2004) have been identified in EST and genomic resources (Lynn *et al*., 2003; Philbin *et al*., 2005; Philbin, 2006). The components that can be identified in the sequence resources include co-receptors (e.g. CD14, dectin-1, MD2), adapter proteins (e.g. MyD88, Tirap, TRIF), signalling intermediates (e.g. TRAF6, IRAK, MAPK (mitogen-activated protein kinase)) and associated transcription factor components (e.g. NFκB, IRF7). To date little confirmation of the precise pathways initiated by chTLR has been completed, although it is predictable that the gross features of chTLR signalling will be conserved with other avian species. This statement is supported by the pharmacological inhibition of oxidative burst in heterophils (Farnell *et al*., 2003a, b) and NO production with the macrophage-like cell line, HD11 (Crippen, 2006).

TLR1/6/10 Related Molecules

In humans, TLR1, 6 and 10 represent a closely related group of TLR receptors. Functionally, TLR1 and TLR6 have been demonstrated to form heterodimers with the related molecule TLR2, influencing the agonist-driven response (with bacterial peptidoglycan) and providing a degree of specificity as seen with recognition of diacyl peptides (TLR1) or triacyl peptides (TLR6; Takeuchi *et al*., 1999; Ozinsky *et al*., 2000). In the chicken, it is clear that at least one TLR1/6/10-like molecule exists (with 44–46% amino acid identity with all three human molecules), which is broadly expressed in tissues and different cell types (Iqbal *et al*., 2005a). Analysis of the draft chicken genome sequence suggested the existence of a second TLR1/6/10-like molecule with very high sequence identity to the previously identified chTLR1/6/10 (Yilmaz *et al*., 2005). However, these sequences remain in the unassigned sequence contigs of the draft genome and this region would benefit from more detailed analysis. No functional studies have been reported for these molecules. Recently, Keestra *et al*. (2007) demonstrated that one chTLR1/6/10-like molecule (termed TLR16) enhanced the ability of chTLR2.2 to respond to both diacylated and triacylated agonists. Creation of chimaeric receptors with exchange of chTLR1/6/10 LRR 6-16 into human TLR6 conferred dual specificity and species-specific interactions with chTLR2.2. In contrast, Higuchi *et al*. (2007) described two chTLR1/6/10-like molecules and reported that combination of TLR1.1 or TLR1.2 with either TLR2.1 or TLR2.2 afforded specificity to the agonist response. The TLR16 of Keestra *et al*. (2007) and the TLR1.1 of Higushi *et al*. (2007) most likely represent the same molecule.

TLR2

In mammals, the TLR2 molecule has been demonstrated to respond to a wide variety of PAMP including bacterial lipoproteins, aribinomannan, peptidoglycan, fungal zymosan and GPI anchors from protozoan parasites (Takeuchi *et al*., 1999; Underhill *et al*., 1999a, b; Almeida *et al*., 2000; Opitz *et al*., 2001; Massari *et al*., 2002; Gazzinelli *et al*., 2004). As indicated in the preceding section, the spectrum of mammalian TLR2 agonists is influenced by the capacity to function as homodimers or as heterodimers with TLR1 or TLR6. The two chTLR2-like molecules have 88.5% nucleotide identity to each other and ~50% amino acid identity to human TLR2. chTLR2 genes are tandemly arranged on chromosome 4, in a region with conserved synteny with the mammalian TLR2-containing genomic regions (Boyd *et al*., 2001; Fukui *et al*., 2001). The two forms of chTLR2, termed type 1 and type 2, are differentially distributed in tissues and cell populations (Fukui *et al*., 2001; Iqbal *et al*., 2005a). ChTLR2 type 1 has more restricted distribution being highly expressed only in spleen, caecal tonsils and liver tissues, and most intensively expressed on heterophils, the DT40 B-cell line and the macrophage-like HD11 cell line (but not on blood monocyte-derived macrophages). In contrast, chTLR2 type 2 is more broadly distributed (Fukui *et al*., 2001; Iqbal *et al*., 2005a).

The oxidative burst activity of heterophils in response to lipoteichoic acid (from *S. aureus*) is inhibited (25–30%) by application of a goat anti-human TLR2 or anti-human CD14 antisera (Farnell *et al*., 2003c). Evidence for conservation of the signalling pathways with TLR2-mediated responses in avian cells is evident from studies on the pharmacological inhibition of the heterophil oxidative burst (Farnell *et al*., 2003c).

TLR3

The TLR3 agonist, dsRNA was widely known as an immunostimulatory compound in both mammals and birds, long before the discovery of TLR molecules – e.g. with chickens (see Becker and Knight, 1990). Human and chicken TLR3 share 58% overall amino acid identity and are expressed in a wide range of tissues and cell populations (Iqbal *et al*., 2005a). The chTLR3 gene is located on chromosome 4 in the chicken, again in a region of conserved synteny with mammalian TLR3 genes (Y. Boyd, personal communication and confirmed by subsequent analysis of the draft chicken genome sequence). In mammals the response to TLR3 agonists is typified by upregulation of type I IFNs and exposure of chicken splenocytes to the synthetic dsRNA, poly inositol cytosine (IC), stimulates upregulation of IFNα and IFNβ mRNA (Philbin *et al*., 2005). The cellular responses to poly IC also includes induction of an oxidative burst and degranulation response in isolated chicken heterophils (Kogut *et al*., 2005b) and production of NO in the macrophage cell line, HD11 (Crippen, 2006). Pharmacological inhibition studies and analysis of phosphorylation status of various signalling components support the general finding that the TLR pathways are conserved between chickens and mammals (Kogut *et al*., 2005b; Crippen, 2006).

TLR4

The response of TLR4-intact cells to LPS exposure is typified by high levels of NO production and expression of the pro-inflammatory cytokines including TNFα and IL-1β. Different mammalian species react more, or less, intensely to the effects of LPS although with most mammals B cell proliferation is induced by exposure to LPS. This effect is not seen with chickens, except at very high doses of LPS (Tufveson and Alm, 1975). Chickens are relatively resistant to systemic administration of LPS (Adler and DaMassa, 1979) and, although the nature of this resistance is unclear, the lack of B cell proliferation may be a contributory factor.

Studies examining the effect of LPS on chicken cells (e.g. Qureshi and Miller, 1991; Miller and Qureshi, 1992; Sunyer *et al*., 1998; Qureshi *et al*., 2000; Dil and Qureshi, 2002a, b, 2003; Farnell *et al*., 2003c) pre-date the formal identification of chTLR4 (Leveque *et al*., 2003). Early efforts to identify a TLR4-mediated response relied upon the cross-reactivity of antibodies raised against human TLR4 which interfered with LPS-mediated induction of NO synthesis, inducible nitric oxide synthase (iNOS or NOS II) mRNA and the cytokines, IL-1β and IL-6 mRNA (Dil and Qureshi, 2002a; Farnell *et al*., 2003c). The chTLR4 gene encodes a protein with 44% amino acid identity to human TLR4 and is located on the micro-chromosome 17 (Leveque *et al*., 2003). Sequence polymorphisms in chTLR4 were detected in several inbred lines of white leghorn chicken and inheritance of the TLR4 locus was associated with survival after exposure of chicks to systemic infection with *S. enterica* serovar Typhimurium (Leveque *et al*., 2003). As with mammalian TLR4 (Zarember and Godowski, 2002), chTLR4 mRNA is expressed in a wide range of tissues, immune cell populations, stromal cells; it is particularly highly expressed on macrophages and heterophils (Leveque *et al*., 2003; Iqbal *et al*., 2005a; Kogut *et al*., 2005b) The expression level of anti-human TLR4 reactivity on chicken blood monocyte-derived macrophages also differs between lines of chicken and levels were correlated with the ability of the cells to produce NO in response to LPS (Dil and Qureshi, 2002b). Hence, chTLR4-mediated LPS responsiveness may be affected by both sequence polymorphism and by amounts of protein expressed on the surface of target cells.

TLR5

The TLR5 molecule responds to flagellin, the most abundant building block of bacterial flagella and clear TLR5 orthologues have been reported in mammals, fish and chickens. The chTLR5 gene is located on chromosome 3 in the chicken genome and shares 50% amino acid identity with human TLR5 (Iqbal *et al*., 2005b). The tissue distribution of chTLR5 mRNA is as extensive as mammalian TLR5 and is found in both immune and stromal cell types (Zarember and Godowski, 2002; Iqbal *et al*., 2005a, b). Exposure of chTLR5$^+$ cells to purified flagellin led to an upregulation in IL-1β mRNA levels and the magnitude of the response correlated with the relative levels

of chTLR5 mRNA detected by RT-PCR. In these experiments, no change was seen with chIL6 mRNA in any of the cell types tested (Iqbal *et al*., 2005b) contrasting with the IL6 detected after stimulation of mammalian TLR5 under similar conditions (e.g. Gewirtz *et al*., 2001).

TLR7 and TLR8

Careful analysis of the TLR7/8 locus has revealed that chTLR7 is present (with 62% amino acid identity to huTLR7) but chTLR8 is fragmented and disrupted by a large CR1-containing insertion. The TLR8-CR1 disruption can be identified in the genomic DNA of a wide range of galliform birds but not in the genomes of diverse groups of non-galliform birds. The implications for this finding are difficult to ascertain but both TLR7 and TLR8 respond to a series of "synthetic modified nucleotide" or virus-associated single-stranded RNA agonists, hence the differential susceptibility of different bird species to viral infections may relate to the disruption of TLR8. In humans, TLR7 and TLR8 exhibit differential agonist specificity and are expressed on different cell types, suggesting distinct function (Hornung *et al*., 2002; Zarember and Godowski, 2002; Heil *et al*., 2003).

The tissue and cellular distribution of chTLR7 mRNA is relatively focused and similar to that described for mammals (Applequist *et al*., 2002; Zarember and Godowski, 2002; Iqbal *et al*., 2005a; Philbin *et al*., 2005), with most TLR7 mRNA being detected in B cells. The mammalian plasmacytoid DC is well defined as a strong responder cell population to TLR7/8 agonists and it is likely that at least some chicken DC subsets will have a similar capacity but the paucity of reagents which stimulate differentiation of chicken DC has precluded this type of analysis. Nonetheless, chTLR7 mRNA is present at lower levels in other cell subsets, including blood-derived heterophils, which also respond to exposure with TLR7-agonists (Kogut *et al*., 2005b). Similar observations have been reported with murine eosinophils (Nagase *et al*., 2003). Although, very little TLR7 mRNA can be detected in fresh blood monocyte-derived macrophages the macrophage-like cell line HD-11 is strongly positive (using RT-PCR) and responds efficiently to application of the agent R848 (Philbin *et al*., 2005). The specificity of chTLR7 for the mammalian TLR7 agonists is supported by transfection studies into TLR7-ve cells (V.J. Philbin and A.L. Smith, unpublished data).

Exposure of chicken splenocytes or HD11 cells to a wide range of TLR7/8 agonists-induced upregulation of a range of cytokine mRNA in a dose-dependent fashion. These include chIL-1β, chIL6, chIL8 but not chIFNα or chIFNβ (Philbin *et al*., 2005) as well as increased levels of NOS II mRNA. In contrast, with purified heterophils, loxoribine exposure downregulated the levels of chIL-1β, chIL6 and chIL8 (CXCL8) mRNA (Kogut *et al*., 2005b) although respiratory burst activity and degranulation were increased by this treatment. The lack of type I IFN response was somewhat surprising, since mammalian peripheral blood lymphocytes respond to TLR7/8 agonists by upregulation of these molecules which are considered important in early defence against viral infections (Lee *et al*., 2003). Moreover, exposure of chickens or chicken splenocytes to the imidazoquinolinamine, S-28828, led to an increase in type I IFN-like activity as measured by bioassay (Karaca *et al*., 1996). The apparent discrepancy in type I IFN production may be resolved by the differential activity of different TLR7 agonists, differences in experimental systems (e.g. *in vivo* or *ex vivo* application will interact with different cell subsets), the response of different cell types or differences in the lines of chicken tested. Nonetheless, the implications of reduced type I IFN responses after TLR7 activation are potentially very important in protection against a wide range of viral pathogens and deserve further investigation.

TLR9

Although it is clear that chickens respond to the classical TLR9 agonist based on a short unmethylated CpG motif (Yang *et al*., 1998; Vleugels *et al*., 2002; He *et al*., 2003, 2005a, b; Kandimalla *et al*., 2003; Wang *et al*., 2003; Dalloul *et al*., 2004; Ameiss *et al*., 2006; Gaunson *et al*., 2006; Loots *et al*., 2006) there is no evidence at present that identifies an orthologue of mammalian TLR9 using EST or genomic sequence resources. Indeed, although conserved synteny is a feature of many TLR-encoding loci, bioinformatic and experimental examination of

the appropriate region of genomic sequence indicates conserved organization of ALAS-1 and PTK9L genes that flank TLR9 in humans and mice, there is no chTLR in this region of the genome (M. Iqbal, V.J. Philbin and A.L. Smith, unpublished). The responses of chicken heterophils, monocytes or peripheral blood leukocytes to application of CpG DNA include production of NO, upregulation of IL-1β and IFNγ (He *et al*., 2003; 2005a, b, 2006; Loots *et al*., 2006) consistent with the activity of a PRR. The functional data with CpG motifs indicate that birds respond to the appropriate TLR9 agonist and chTLR9 may be present in a non-syntenic region of the chicken genome or, alternatively, recognition of the CpG motif may occur via a different PRR.

Avian TLR Without Mammalian Orthologues; chTLR15 and TLR21

Two TLR genes can be located in the chicken genome, chTLR15 (chromosome 3) and chTLR21 (chromosome 11; Roach *et al*., 2005; Higgs *et al*., 2006; A.L. Smith, V.J. Philbin and R. Beal, unpublished data). The latter has been referred to as chTLR21 because of sequence homology with fish TLR21 but, as indicated, below this notation should be used with caution.

Initial characterization of chTLR15 indicates that this molecule is not orthologous to any known TLR molecule in mammals or fish (Roach *et al*., 2005; Higgs *et al*., 2006) but it may loosely group with the TLR1/6/10 and TLR2 molecules in phylogenetic analysis (Higgs *et al*., 2006). The expression of chTLR15 mRNA was highest in bone marrow, bursa and spleen and expression in the caecum was increased by infection with *S.* Typhimurium, as was chTLR2 mRNA, suggesting that these TLR may be found on cells infiltrating into the gut (perhaps even on the same cell subset). Moreover, exposure of chicken primary embryonic fibroblast cultures to heat-killed salmonella led to upregulation of chTLR15 mRNA, which may be the result of activation of other chTLR constitutively present on the cultured cells.

A further TLR gene was included in a broad analysis of vertebrate TLR evolution and termed chTLR21 due to amino acid sequence homology with *Fugu* TLR21 (Roach *et al*., 2005). However, the level of amino acid identity with *Fugu* TLR21 and TLR22 are very similar at 44% and 38%, which is reminiscent of the level of identity seen between different members of a TLR subfamily. For example, whereas the chTLR7 amino acid sequence is 62% identical to huTLR7, it is also 40% and 35% to huTLR8 and huTLR9 which are also members of this TLR subfamily. The distribution of LRR which decorate the extracellular region of the TLR is generally conserved between orthologous TLR from different species. Analysis of the putative chTLR21 reveals significant divergence from predicted LRR within the fish TLR21 and TLR22 molecules (V.J. Philbin, R. Beal and A.L. Smith, unpublished) hence, to avoid mistaken attribution of an orthologous link between these TLR, we propose that this chTLR be given a unique identifier. The expression of chTLR21 mRNA can be detected in a wide range of tissues from adult white leghorn chickens including the spleen, bursa and gut (R. Beal, V.J. Philbin and A.L. Smith, unpublished) but further analysis is required before the contribution of this chTLR to the avian immune system can be defined.

TLR Signalling Pathways in Chickens

The consequences of TLR activation are diverse and include activation of cytokine, IFN and chemokine production, cell maturation, induction of degranulation and the direct production of AMP. The literature covering the effects of TLR activation in mammalian systems is vast, whereas much more limited information is available with avian systems. For detailed coverage of signalling cascades associated with TLR activation, the reader is referred to recent reviews (Medzhitov and Janeway, 2000; O'Neill and Dinarello, 2000; Akira and Takeda, 2004) and general aspects will only be covered here. The optimal activation of at least some TLR involves the co-ordinated action of a variety of molecules which has been most intensely studied with TLR4–LPS interactions and shown to include the LBP, CD14 and MD2 molecules. Genes encoding orthologues of each of these molecules can be identified in the chicken genome resources based on sequence identity, gene structure and conserved synteny (V.J. Philbin and A.L. Smith, unpublished; P. Kaiser, personal communication).

Activation of the TLR leads to recruitment of adapter proteins to the TIR domain and for most TLR these include the MyD88 adapter, however, with TLR3 the only known adapter mediating signal transduction is TRIF. With TLR4 it is clear that a wide range of adapters are involved with signal transduction including MyD88, TIRAP, TRIF, TRAM and SARM and it is likely that each configuration of adapter molecules invokes different downstream modifications to the signalling cascade. For example, with TLR4, recruitment of TRAM to the TIR domain leads to interaction with TRIF resulting in induction of type I IFN responses (Oshiumi *et al*., 2003). The downstream components of TLR signalling cascades in mammals include IRAK-1, IRAK-4, TAK-1, TRAF6, MAPK pathway elements, ESCIT, BTK, IκB kinases, NFκB and IRF family members. Very little work has been completed in chickens although many of the elements of these pathways are clearly conserved and can be identified using EST and genomic resources (Lynn *et al*., 2003; Philbin *et al*., 2005; V.J. Philbin and A.L. Smith, unpublished observations). Further evidence for the conservation of TLR signalling requirements in chickens and mammals comes from the conserved effects of a range of pharmacological inhibitors of heterophils and the HD11 macrophage-like cell line (Farnell *et al*., 2003a, b; Crippen, 2006). Nonetheless, there also appear to be some species-specific differences in the consequences of TLR activation, as judged by the analysis of cytokine and IFN production with chTLR5 and chTLR7 (Iqbal *et al*., 2005b; Philbin *et al*., 2005).

General Considerations in Pattern Recognition

PRR can be subdivided according to the locality of PRR–PAMP interactions into those that operate in body fluids or those that are cell-associated; the latter can be further subdivided in terms of monitoring the external mileau (e.g. TLR) or cell cytoplasm (e.g. NODs, PKR). Moreover, many of the PRR that monitor different areas in the host recognize related groups of PAMP such as seen with bacterial LPS and viral dsRNA, which provides the host with efficient pathogen detection in multiple compartments. For example, the capacity to detect intracellular pathogens within the cytoplasm of the first infected cell, in neighbouring cells and systemically, during pathogen translocation between cells, provides a multi-layered recognition/defence system. The cellular responses to PRR stimulation differ considerably, dependent on the cell type and the context of PAMP exposure. With chickens this can be seen with the differential responses to TLR5 and TLR7 agonists in heterophils (Kogut *et al*., 2005b) and other cell types (Iqbal *et al*., 2005b; Philbin *et al*., 2005). Moreover, any individual pathogen type usually presents a variety of PAMP structures to the host and the form taken by the developing immune response is, at least partly, driven by the combined effects of a range of PRR signals.

SUMMARY

The innate immune responses in the chicken are a key to understand host/pathogen interactions. TLR-mediated antigen recognition is a key event with important consequences for the adaptive immune response. Innate immune responses are thus essentially involved in orchestrating and modulating the immune response towards a pathogen. Most of these aspects of the chicken innate immune system have been characterized in the last 10 years.

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8 THE AVIAN MHC

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INTRODUCTION
THE CLASSICAL CHICKEN MHC IS SMALL, SIMPLE AND REARRANGED
THE CLASSICAL CHICKEN MHC ENCODES SINGLE DOMINANTLY-EXPRESSED CLASSICAL CLASS I AND II MOLECULES
THE PROPERTIES OF THE SINGLE DOMINANTLY-EXPRESSED CLASS I AND II MOLECULES CAN EXPLAIN RESPONSES TO PATHOGENS AND VACCINES
THE SINGLE DOMINANTLY-EXPRESSED CLASS I MOLECULE IS DUE TO CO-EVOLUTION WITH TAP AND TAPASIN
THE CHICKEN MHC GIVES INSIGHTS INTO THE PRIMORDIAL MHC AND THE SUBSEQUENT EVOLUTION OF THE MHC
REFERENCES

INTRODUCTION

The major histocompatibility complex (MHC) was discovered in mice as the genetic locus responsible for rapid tissue allograft rejection, and found to encode a number of highly polymorphic alloantigens on the surface of cells. It is now known that graft rejection is an accidental consequence of the basic function of the polymorphic alloantigens, the MHC classical class I and II molecules, whose role is to bind and present peptide fragments to T lymphocytes of the immune system. Most detailed understanding of the structure and function of the MHC and MHC molecules comes from work with humans and mice (Janeway *et al.*, 2005).

The class I and II molecules have a similar overall structure but the domains are connected differently. The class I molecules consist of a transmembrane glycoprotein heavy chain, non-covalently associated with a small non-polymorphic immunoglobulin (Ig) constant-like protein, β_2-microglobulin, which is encoded on a different chromosome. Mature class I heavy chains consist of two "open-faced sandwich" domains (α1 and α2) followed by an Ig constant-like domain (α3), a connecting peptide, transmembrane region and cytoplasmic region. The class II molecules consist of two non-covalently associated glycoprotein chains (α and β chains encoded by A and B genes), each of which consists (in the mature form) of an "open-faced sandwich" domain (α1 and β1) followed by an Ig constant-like domain (α2 and β2), a connecting peptide, transmembrane region and cytoplasmic region. Thus, the extracellular regions of both class I and II molecules have two "open-faced sandwich" domains atop two Ig constant-like domains. In classical class I and II molecules, the open-faced sandwiches, which together form a groove with two α-helices atop a β sheet, bind peptides in the groove where most of the polymorphic amino acids are located.

Classical class I molecules bind peptides primarily derived from proteins found in the cytoplasm, while classical class II molecules bind peptides primarily derived from proteins in intracellular vesicles and taken up from the extracellular space. The classical class I molecules are found on

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almost all cells, and are recognized by CD8-bearing T cells, most usually cytotoxic T lymphocytes (CTL; described in Chapter 5). CTL kill transformed cells and virally infected cells (as well as those cells infected with bacteria that reside in the cytoplasm), preventing replication of tumour cells or release of the pathogen. Classical class II molecules are found mainly on antigen-presenting cells (APC; described in Chapter 9), and are recognized by CD4-bearing T cells, usually helper T cells, which induce or permit immune responses including the production of antibodies to extracellular pathogens.

The high level of polymorphism in classical MHC molecules is thought to be due to a molecular arms race with pathogens, in which a particular pathogen escapes T cell recognition by mutating those peptide(s) that bind to an MHC molecule, thus selecting for hosts which have different MHC molecules that bind the peptide(s) of a newly mutated pathogen. Much evidence of different kinds supports the idea that most of the allelic polymorphism and sequence diversity is involved in peptide binding, including the fact that most of the sequence differences are found in the peptide-binding site. In fact, the human MHC is known to have many disease associations, although most are with autoimmune diseases.

There are also non-polymorphic non-classical MHC molecules, not all of which are encoded in the MHC. Some non-classical class I molecules bind conserved antigens for recognition by T cells, for instance special peptides (such as H-2M3-binding bacterial formyl-methionine peptides or H-2Qa1/HLA-E binding signal sequences) or lipids (such as CD1 binding mycobacterial glycolipids). Other non-classical class I molecules are recognized by special receptors (such as stress-induced MIC and Rae molecules recognized by natural killer (NK) cell receptors). Still other non-classical class I molecules are not involved in immune recognition of antigens, but have a range of functions including antibody binding (neonatal Fc receptor), iron binding (haemochromatosis gene), lipid transport (Zn-α_2-macroglobulin), neural patterning (H-2M5) and so on. Non-classical class II molecules (DM and DO) are involved in peptide loading of classical class II molecules in a special intracellular vesicle, the MIIC compartment.

The mammalian MHC is an enormous region, both in physical and genetic distances, with many genes and pseudogenes as well as much repetitive DNA. The typical structure is divided into three regions. The class I region contains many classical and non-classical class I genes which vary from one species to another, as well as other kinds of genes spread out in a framework common to many mammals. The class II region is much more conserved between mammals, and contains several pairs of classical class II A and B genes, the non-classical DM and DO genes involved in peptide loading of class II molecules, some genes involved in peptide loading of class I molecules including the interferon-inducible proteasome genes (LMP), the transporter involved in antigen presentation genes (TAP) and the TAP-associated protein (tapasin), as well as the mysterious nuclear serine/threonine protein kinase (RING3) and other assorted genes. In between the class I and II regions is the highly conserved class III region, which contains a whole assortment of different kinds of genes, notably including some components of the complement cascade (C2, factor B (fB) and C4) and cytokine genes of the tumour necrosis factor (TNF) family (TNF and LT (lymphotoxins)).

In contrast to the enormous body of knowledge about the mammalian MHC, particularly in humans and mice, much less is known about the MHC in non-mammalian vertebrates (Flajnik and Kasahara, 2001; Kelley *et al*., 2005). Apparently, the MHC arose about the same time as the rest of the adaptive immune system at the base of the vertebrate tree. The genes for MHC class I and II molecules, TAPs, tapasin, α, β, γ and δ T cell receptors, antibodies and recombination-activating gene (RAG) molecules are all found in amphibians, bony fish and cartilaginous fish but not in the jawless fish, such as lampreys and hagfish. The chicken MHC was originally identified as the B blood group (Briles *et al*., 1950; Gilmour 1959; Schierman and Nordskog, 1961), and shown to determine resistance to certain infectious pathogens (Hansen *et al*., 1967). Ever since its discovery, the characterization of the chicken MHC has been at the forefront of our understanding of the structure, function and evolution of the MHC as understood from non-mammalian vertebrates.

THE CLASSICAL CHICKEN MHC IS SMALL, SIMPLE AND REARRANGED

While the draft sequence of the chicken genome was an outstanding accomplishment (International Chicken Genome Sequencing Consortium, 2004), the sequences for the chromosome on which the MHC is located were particularly poorly-assembled, so that virtually nothing new was learned about the chicken MHC. However, the sequence of the region that contains the classical MHC was already published (MHC Sequencing Consortium, 1999), with other regions, loci and genes on the same chromosome understood at lower levels of resolution.

The nucleolar organizing region (NOR), which contains many ribosomal RNA genes, was located to chicken chromosome 16 (a microchromosome) by a silver staining technique. The B locus was shown to be on the same chromosome by use of chromosome 16-triploid animals (Bloom and Bacon, 1985). The B locus was separated into two regions by recombination, the BF/BL region containing the classical MHC and the BG region which determines the BG antigens on erythrocytes (Pink *et al*., 1977; Vilhelmova *et al*., 1977; Simonsen *et al*., 1980). Importantly, no recombination has been observed within the BF/BL region for any experimental mating, and only one "natural recombinant" leading to the Scandinavian B19 (re-designated B19v1) has been found (Hala *et al*., 1979, 1988; Simonsen *et al*., 1982; Koch *et al*., 1983; Skjødt *et al*., 1985). Another locus, dubbed the Rfp-Y region, was identified to contain at least one polymorphic non-classical class I gene along with non-classical class II B genes and lectin-like genes. The Rfp-Y locus has been located to the same microchromosome as the B locus but at sufficient genetic distance to be unlinked, perhaps separated by the highly repetitive and thus recombinogenic NOR (Briles *et al*., 1993; Miller *et al*., 1994a, b, 1996; Afanassieff *et al*., 2001; Rogers *et al*., 2003). Three other loci have been mapped to this microchromosome: BLA encoding the class II A chain located 5 cM away from the BF/BL region (Salomonsen *et al*., 2003), fB encoding complement factor B located some 12 cM from the Rfp-Y region (Kaufman *et al*., 1999a) and G9 (BAT8) from the class III region linked to the BG region (Spike and Lamont, 1995). The order and orientation of some of these loci are as yet unknown.

The sequence of the BF/BL region of the B12 haplotype showed that the chicken MHC is smaller, simpler and organized differently compared to the MHC of typical mammals (Fig. 8.1), as originally predicted by Pink, Ziegler and co-workers on genetic evidence and then by Guillemot, Auffray and co-workers on molecular genetic evidence (Ziegler and Pink, 1976; Pink *et al*., 1977; Guillemot *et al*., 1988; Kaufman *et al*., 1999a, b). Long distance PCR and sequencing throughout the classical MHC has confirmed the same organization for another six haplotypes in nine chicken lines (Kaufman *et al*., 1999a; Jacob *et al*., 2000; Shaw *et al*., 2007). Four main points emerged from this work. First, the BF/BL region is simple and compact, with only 11 genes in the 44 kB of the central region spanning the class I and II B genes and only 19 genes in the sequenced 92 kB. Second, some of the genes present in the MHC of typical mammals are found in this region (such as class I, II B, TAP, DM, RING3 and C4 genes) but many are absent (including class II A, LMP, DO, C2/fB and other class III region genes). Third, the chicken genes are organized differently compared to the mammalian MHC, with the TAP genes flanked by the class I genes, the tapasin gene flanked by class II B genes, and the class III region genes represented by the complement component C4 outside of the class I and II region genes. Fourth, there are genes present in the sequenced region that would not be expected, based on the MHC of typical mammals (including the C-type animal lectin membrane protein genes, and the BG gene for which there is no mammalian orthologue). All of these points have important implications considered below.

More recently, several other genes have been identified flanking the sequenced BF/BL region, along with one of the sequenced genes being more precisely identified (Fig. 8.1): centromere protein A (CENP-A), tenascin, steroid-21 monooxygenase gene (CYP-21), and CD1 genes on one side and TRIM-B30.2 and BG genes on the other (Regnier *et al*., 2003; Maruoka *et al*., 2005; Miller *et al*., 2005; Ruby *et al*., 2005; Salomonsen *et al*., 2005). In mammals, the class I like CD1 genes are located in a so-called paralogous region on a non-MHC chromosome (for instance, human chromosome 1), along with many other genes related to those found in the MHC. In contrast, two chicken CD1 genes were identified about 50 kbp beyond the class I gene

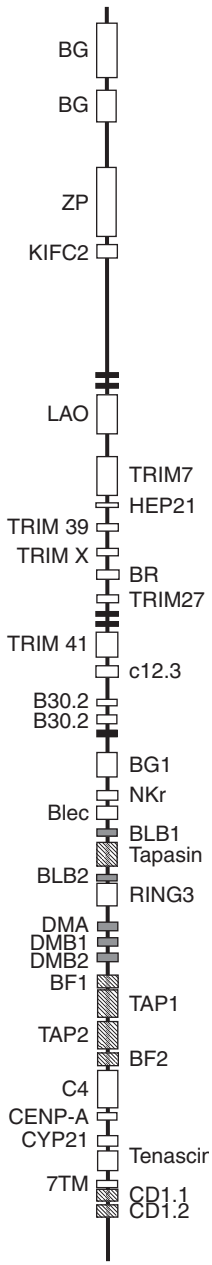

FIGURE 8.1 Region of the B locus currently sequenced includes the BF/BL region and part of the BG region. Genes represented by boxes: black boxes indicate tRNA genes, a zinc finger gene and an uncharacterized gene; lined boxes indicate genes that are part of the class I presentation system and stippled boxes indicate genes that are part of the class II presentation system. Names of genes on right-hand side indicate transcription from top to bottom, on left-hand side from bottom to top. Sequence data and identifications from Guillemot *et al.* (1988) Guillemot *et al.* (1989), Kaufman *et al.* (1999b), Maruoka *et al.* (2005), Regnier *et al.* (2003), Ruby *et al.* (2005), Salomonsen *et al.* (2005) and from the draft chicken genome sequence accessed at www.ensembl.org/Gallus_gallus. This organisation has now been confirmed by sequencing BAC's (Shiina *et al.*, 2007).

and C4 genes. In between are located the CENP-A gene (found on human chromosome 2, and originally identified in the sequenced BF/BL region as a histone gene), the CYP-21 and tenascin genes (both found in the class III region of mammals). TRIM (tripartite motif)-B30.2 genes are found in a variety of locations in the mammalian genome. Six TRIM-B30.2 genes were located 20–70 kbp beyond the BG gene of the BF/BL region. In between the TRIM-B30.2 genes and the

BG gene are a leu-tRNA and two B20.2 genes sequenced as part of the BF/BL region, and the c12.3 gene which encodes a guanine nucleotide-binding protein gene (Guillemot *et al*., 1989). Two of the TRIM-B30.2 genes are orthologous to those located in the extended class I region of the human MHC, two are orthologous to genes on human chromosome 5 next to the gene orthologous to the c12.3 gene and two are not well related to any particular human TRIM-B30.2 gene. One BG gene is located in the BF/BL region, but Southern blots of DNA from recombinants show that many homologous genes are located in the BG region (Kaufman *et al*., 1989, 1999a, b). This region has been located beyond the TRIM-B30.2 locus by Southern blots of bacterial artificial chromosome (BAC) contigs (Ruby *et al*., 2005), and some 15 expressed BG genes have been identified in the B12 haplotype (J. Kaufman, K. Skjødt and J. Salomonsen, unpublished). While there are no reported mammalian orthologues of the chicken BG genes, they are related to genes of the butyrophilin family, at least eight members of which are present at the junction of the class II and III regions and in the extended class I region of the human MHC (Stammers *et al*., 2000; Rhodes *et al*., 2001).

Many genes expected in the MHC are not found in or near the chicken BF/BL region, and are moved elsewhere or may be missing from the chicken genome altogether. For instance, in mammals and many other vertebrate species, the MHC class III region contains two pairs of complement component genes duplicated in tandem: two C4 genes along with fB and C2. In chickens, there is one C4 gene located in the BF/BL region but another is located on chicken chromosome 1 (Kaufman *et al*., 1999a, b; International Chicken Genome Sequencing Consortium, 2004; Skjødt, K., Marston, D., Dodds, A., Bumstead, N., Croojimans, R., Goodchild, M., Powell, T., Rogers, S. and Kaufman, J., in preparation). The chicken fB gene is located 12 cM away from the Rfp-Y region, while the C2 gene is located on chicken chromosome 20 (Kaufman *et al*., 1999a; International Chicken Genome Sequencing Consortium, 2004; Vitved, L., Marston, D., Bloom, S., Bumstead, N., Croojimans, R., Goodchild, M., Holmskov, U., Koch, C., Rogers, S., Salomonsen, J., Teisner, B., Skjødt, K. and Kaufman, J., in preparation). No genes expected from the MHC are located close to these chicken complement genes on chromosomes 1 and 20, so they do not appear to be in MHC-paralogous regions. Thus far, no trace of genes orthologous to the TNF and LT genes present on one end of the human MHC class III region have been found, either in the draft chicken genome sequence or in the databases of expressed sequence tags (EST). However, no EST for Th2 cytokines were found before their genes were cloned by synteny, and a potential TNF type-1 receptor gene has been found (Kaiser *et al*., 2005), so it may be that the TNF and LT genes are indeed present but remain to be identified. Also found in all vertebrates examined are inducible proteasome components genes (originally named LMP2 and LMP7) which in mammals are located in the MHC class II region, but no trace of these have been discovered in chickens. Many other genes from the class III region as well as the extended class I and II regions also remain to be documented.

In summary, there are three major points that emerge from all of the work so far. First, the classical chicken MHC is simpler and more compact than that of mammals, and is not arranged the same as mammals. Second, the classical MHC is flanked by regions containing some of the genes that might have been expected, based on the typical mammalian MHC, but many other expected genes have moved elsewhere (or may be gone from the genome altogether). Third, the classical MHC contains, and is flanked by, some unexpected genes, which are found elsewhere in the mammalian genome. Various arguments considered below suggest that in many (but not all) cases it is the mammalian MHC that has suffered changes compared to that of ancestral non-mammalian vertebrates.

THE CLASSICAL CHICKEN MHC ENCODES SINGLE DOMINANTLY-EXPRESSED CLASSICAL CLASS I AND II MOLECULES

The sequence of the BF/BL region of the B12 haplotype confirms the preliminary organization based on Southern blots and cDNA isolation, that there are two class I genes (originally called BFI and BFIV, or BF minor and BF major, now called BF1 and BF2; Miller *et al*., 2004) in opposite transcriptional orientation flanking the TAP1 and TAP2 genes that are also in opposite transcriptional orientation (Guillemot *et al*., 1988; Kaufman *et al*., 1995, 1999a, b). Long distance

PCR and sequencing has confirmed the same organization for another six haplotypes in nine chicken lines, except for the B14 and B15 haplotypes, for which the BF1 gene has suffered significant but as yet undefined changes (Shaw *et al*., 2007).

Cloning of cDNA confirms that there are two classical class I sequences in many common MHC haplotypes from egg-layer lines (Kaufman *et al*., 1995, 1999a; Juul-Madsen *et al*., 2000; Wallny *et al*., 2006; Shaw *et al*., 2007). However, there was a substantial difference in the number of cDNA clones: only BF2 clones were found for B14 and B15, and as much as 10-fold more BF2 clones than BF1 clones for B2, B4, B12, B19 and B21. This led to the interim nomenclature of major (BF2) and minor (BF1) class I genes. Sequencing of the whole genes showed that all of the BF2 genes were intact, and that the promoters were intact and nearly identical. In contrast, the BF1 genes from B14 and B15 were disrupted, and the promoters of the other BF1 genes were modified: a large deletion encompassing the enhancer A site for B12 and B19, and divergence in the enhancer A site as well as small deletions in the transcriptional start sites for B2, B4 and B21 (Kaufman *et al*., 1999a; Shaw *et al*., 2007). In commercial broiler lines, amplification gave partial genomic sequences for two classical class I genes, one of which had fewer alleles, similar promoter defects and in one case a splice site mutation (Livant *et al*., 2004; Lima-Rosa *et al*., 2004). Thus, there are at least four independent events which have disabled the BF1 gene. Two dimensional gel electrophoresis of pulse-labelled protein immunoprecipitated by a monoclonal antibody to the invariant chain β_2-microglobulin showed that only one class I chain is present in substantial amounts in B4, B12 and B15 peripheral blood lymphocytes. Isolation of bound peptides from B4, B12 and B15 erythrocytes and spleen cells showed that only peptides consistent with binding the BF2 class I molecule were found (Wallny *et al*., 2006). Thus, at least for the haplotypes examined in detail, there is a single dominantly expressed class I molecule at the level of RNA, protein and antigenic peptide.

The BF2 class I molecule has all of the characteristics expected for presentation of antigenic peptides to T lymphocytes. In contrast, the BF1 gene has been disabled in at least three independent ways, is expressed at lower (for many haplotypes much lower) amounts on the cell surface, has far fewer alleles (and some frank pseudogenes) and has less evidence for strong selection on the peptide-binding residues. Moreover, phylogenetic trees of the alleles of BF1 and BF2 (including whole gene sequences) show independent evolutionary histories, with the BF1 gene very similar to the poorly-expressed class II B gene BLB1, suggesting that the evolutionary histories of BF1 and BLB1 largely reflect accumulation of neutral changes in the descent of stable haplotypes (Wallny *et al*., 2006; Shaw *et al*., 2007). Thus, the BF1 gene does not seem to be strongly selected for presentation of antigenic peptides. However, based on a small sequence motif in the α helix of the α1 domain, it has been suggested that the BF1 molecule could function as a ligand for Ig-type NK cell receptors, much as HLA-C does for killer Ig-like NK receptors (KIR) in humans (Livant *et al*., 2004). Recently, a large number of such NK cell receptors in the chicken Ig-like receptor (chIR) locus have been described in chickens (Viertlboeck *et al*., 2005; Laun *et al*., 2006).

Similar to the class I genes, there are two class II B genes (which encode class II β chains) in opposite transcriptional orientation flanking the tapasin gene in the sequence of the B12 haplotype (Guillemot *et al*., 1988; Frangoulis *et al*., 1999; Kaufman *et al*., 1999a, b). Long distance PCR and sequencing has confirmed the same organization for another six haplotypes in nine chicken lines (Jacob *et al*., 2000). Moreover, just like the class I genes, the BLB2 gene is expressed much more than the BLB1 gene at the RNA level (Pharr *et al*., 1998; Jacob *et al*., 2000). A single class II A gene (the BLA gene which encodes the class II α chain) has been mapped 5 cM away from the BF/BL region, and cDNA sequences show it to be a non-polymorphic DR-like gene (Salomonsen *et al*., 2003). Therefore, it seems most likely that there will be a single dominantly expressed class II molecule on the surface of chicken cells.

THE PROPERTIES OF THE SINGLE DOMINANTLY-EXPRESSED CLASS I AND II MOLECULES CAN EXPLAIN RESPONSES TO PATHOGENS AND VACCINES

In humans, the MHC is known to be the genetic region with most associations with disease. However, the strong associations are generally with autoimmune disease or with particular

biochemical defects, rather than with resistance to infectious pathogens (Tiwari and Terasaki, 1985; Hill, 1998). Despite much deliberate examination, there are few examples of strong associations with resistance to infectious pathogens, the best being with HIV progression (Hill, 1998; Carrington *et al*., 1999; Carrington and O'Brien, 2003; Altfeld *et al*., 2006). In contrast, over 50 years ago poultry researchers found very strong genetic determination of resistance and susceptibility to certain economically important pathogens. A particularly important locus determined the B blood group, typed with alloantisera which were likely to have recognized mostly the BG antigens on erythrocytes (Hansen *et al*., 1967).

There are many examples of disease associations with the B locus, including resistance to viral, bacterial and parasitic pathogens (Briles *et al*., 1977; Bacon, 1987; Bacon *et al*., 1981, 2000; Calnek, 1985; Schat, 1987; Dietert *et al*., 1990; Plachy *et al*., 1992, 1994; Cotter *et al*., 1998; Lamont 1998; Hudson *et al*., 2002; Liu *et al*., 2002; Macklin *et al*., 2002; Joiner *et al*., 2005; Schou *et al*., 2007). Some of these associations have been mapped to the BF/BL region, now known to be the classical chicken MHC. In particular, the strong associations of resistance to the tumours caused by the herpesvirus Marek's disease virus (MDV) and the retrovirus Rous sarcoma virus (RSV). In addition, the B locus has strong effects on response to certain vaccines, both live attenuated vaccines and inactivated vaccines (Bacon *et al*., 1987; Heller *et al*., 1991; Juul-Madsen *et al*., 2002, 2006; Liu *et al*., 2002; Zhou and Lamont, 2003). Finally, as in mammals, the B locus is associated with various autoimmune diseases, including autoimmune thyroiditis and vitiligo (Bacon *et al*., 1987; Wang and Erf, 2004; Wick *et al*., 2006); these diseases are described in Chapter 18.

The hypothesis of "the minimal essential MHC of the chicken" attempts to provide a molecular basis for the striking disease associations of the chicken MHC, in comparison to what is known for well-characterized mammalian models (Kaufman *et al*., 1995, 1999a, b; Kaufman, 2000). In this view, the multigene families of class I and II molecules, as well as other disease resistance genes of the huge and complex mammalian MHC, confer more-or-less protection to most pathogens (leading to weak associations with infectious disease); whereas the properties of the single dominantly expressed class I and II molecules of the small and simple chicken MHC confer either resistance or susceptibility to a particular pathogen (leading to strong associations with infectious disease). This can be illustrated (Fig. 8.2) by considering that there is a strong probability that one of the six class I molecules of a typical human (HLA-A, B and C in a heterozygote) will bind a pathogen peptide that confers protection, whereas there is much less chance for the two class I molecules of a typical chicken (BF2 in a heterozygote) to bind a peptide. Moreover, it is also probable that one of the six human class I molecules will

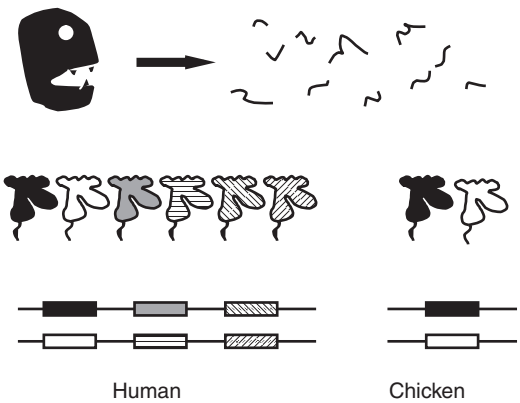

FIGURE 8.2 In comparison to mammals, the chicken MHC has strong associations with resistance and susceptibility to infectious pathogens. A pathogen (upper left) is proteolysed within a cell into peptides (upper right). There is more chance for the six human class I molecules (middle left) arising from three heterozygous gene loci (bottom left) to find a protective peptide than for the two chicken class I molecules (middle right) arising from one heterozygous gene locus (bottom right) (adapted from Kaufman *et al*., 1995).

bind a self peptide, leading to strong associations of particular MHC alleles with autoimmunity. In this view, better MHC-determined protection from pathogens comes at the cost of more autoimmunity.

Peptide motifs have been determined for the class I molecules of a number of common chicken MHC haplotypes, by eluting peptides from class I molecules isolated from erythrocytes and spleen cells, followed by sequencing of peptide pools and individual peptides (Kaufman *et al*., 1995; Wallny *et al*., 2006). For some haplotypes (such as B4, B12, B15 and B19), the peptides were mostly octamers or nonamers, with obvious motifs which correlated well with the binding sites of the dominantly-expressed class I molecule. For instance, the peptides from B4 cells have negatively charged aspartic acid or glutamic acid at positions 2 and 5, and glutamic acid only at position 8. The binding site of BF2*0401 has positively charged arginines in the appropriate places to bind peptides with these negative charged anchor residues. The motifs for the class I molecules from these haplotypes appeared every bit as fastidious as those for human and mouse class I molecules.

These peptide motifs were used to examine the MHC-determined resistance and susceptibility to tumours induced by RSV. This classic acutely transforming retrovirus bears four genes: *gag*, *pol* and *env* in common with other avian leukosis viruses, and *v-src* which was acquired from the host cellular gene *c-src*. A large scientific literature shows that many chicken lines are infected and develop tumours induced by *v-src*, which either progress leading to mortality or regress leading to survival. The MHC is the major response locus for progression or regression, with the combination of viral strain and MHC haplotype determining whether an immune response leads to regression (Plachy *et al*., 1994; Taylor, 2004). In the particular system examined, MHC-congenic strains CB (B12) and CC (B4) infected with RSV Prague strain C, many more peptides were predicted to fit the resistant B12 motif than the susceptible B4 motif (Wallny *et al*., 2006). Binding studies and vaccination with various peptides indicated that the peptide conferring resistance by the B12 haplotype is derived from the *v-src* gene in the only region substantially different from the host *c-src* gene sequence (Kaufman, 2000; Hofmann *et al*., 2003). Thus, whether the single dominantly-expressed class I molecule has a motif to bind a protective peptide can determine life or death from this relatively small and simple pathogen. Analyses of other simple viruses and viral vaccines give similar results.

Many vaccines used in poultry are inactivated, and lead to the production of antibodies, which in mammals is a class II-dependent response. There are several reports showing that the chicken MHC determines responses to inactivated vaccines (Bacon *et al*., 1987; Heller *et al*., 1991; Juul-Madsen *et al*., 2002, 2006; Liu *et al*., 2002; Zhou and Lamont, 2003), which could be explained by the peptide motif of the dominantly expressed class II molecule. Indeed, class II molecules were first identified in mammals as immune-response (*Ir*) genes that determined the antibody response to immunogens with limited epitopes (Kantor *et al*., 1963; McDevitt and Chinitz 1969). What is striking for the chicken is that the same phenomenon is found for huge antigens with many epitopes, such as whole inactivated viruses (Fig. 8.3), presumably because chickens have a single dominantly-expressed class II molecule rather than the multigene family of typical mammals. Some progress has been made towards determining the peptide motifs of chicken class II molecules, with the publication of a motif for the B19 haplotype (Haeri *et al*., 2005; Cumberbatch *et al*., 2006). The peptides bound to class II molecules from several other haplotypes have been determined, and one interesting phenomenon is the presence of a few dominant peptides for certain haplotypes (J. Kaufman, J. Salomonsen, H.-J. Wallny, D. Avila, F. Johnstone, I. Shaw, J. Jacob and L. Hunt, unpublished observations).

Many pathogens, including viruses with relatively large genomes, encode so many proteins that even class I molecules with very fastidious peptide motifs should find protective peptides. One example is MDV, a herpesvirus that encodes at least 80 proteins (Osterrieder *et al*., 2006). A large scientific literature shows that many chicken lines are infected and develop tumours induced by MDV. Several genetic loci that contribute to resistance and susceptibility have been identified in crosses between inbred chicken lines (Vallejo *et al*., 1998). However, despite wide variation in host genetics, sex, age and environment as well as pathogen strain, dose and route

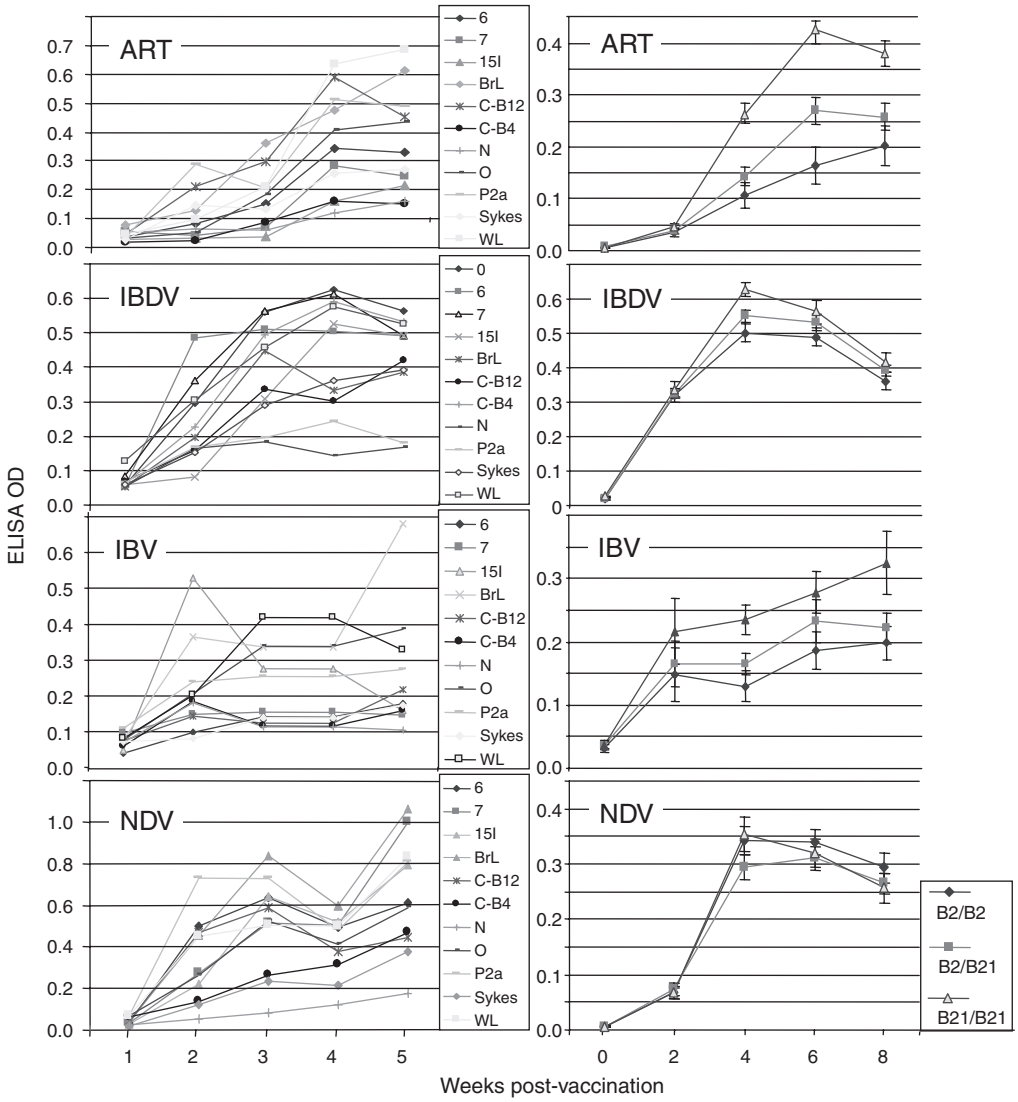

FIGURE 8.3 There are enormous differences in antibody responses by different inbred lines of chickens, which can be determined by the MHC. Left-hand column: six chickens at 18 weeks of age from each of 11 inbred strains (lines 6_1 and 7_2, B^2; C-B4, B^4; C-B12, B^{12}; Wellcome (WL), B^{14}; 15I, B^{15}; P2a, B^{19}; N and O, B^{21}; BrL and Sykes, B haplotype not known in detail) at the Institute for Animal Health (Compton) were immunized once with a commercial vaccine containing four inactivated viruses (ART, avian rhinotracheitis virus; IBDV, infectious bursal disease virus; IBV, infectious bronchitis virus; NDV, Newcastle disease virus), and antibody responses determined by enzyme-linked immunoabsorbent assays (ELISA). Right-hand column: 101 ($N \times 6_1$)F2 chickens were immunized and analysed for antibody responses as above, and typed for B haplotype using a microsatellite marker (unpublished data from G. Heal, J. Kaufman and N. Bumstead).

of infection, most studies find a strong effect of the MHC, with the B19 haplotype usually being the most susceptible, B21 being the most resistant and other common haplotypes in a roughly reproducible rank order in between (Plachy *et al*., 1992).

There are several models for this MHC-determined resistance to MDV. One model is that only the CTL response to peptides from a particular MDV gene is crucial to resistance. Active CTL directed to several MDV proteins are found at low frequencies for both the B19 and B21 haplotypes, but CTL to the ICP4 protein have been found in B21 but not B19 birds (Omar and

Schat, 1996). Another model is that there is at least one (and perhaps several) different forms of "genetic resistance" involving polymorphic MHC genes with mechanisms outside of the innate and adaptive immune systems. One example might be the unexpected affinity between a class II B gene sequence and a viral protein identified by bacterial two-hybrid assays (Niikura *et al*., 2004).

A third model is based on the different levels of class I molecules on the cell surface. This cell surface expression level varies between MHC haplotypes, with the difference as much as 10-fold in certain cell types, and is due to some aspect of transport to the cell surface and not transcription, translation or assembly. The rank hierarchy of expression correlates with susceptibility to Marek's disease (MD) as reported in the scientific literature, with the most susceptible haplotype B19 having the highest level of cell surface expression and the most resistant B21 the lowest (Kaufman *et al*., 1995; Juul-Madsen *et al*., 2000). In fact, there is evidence for the same differences in cell surface expression level for alleles of single class I genes in mammals (Neisig *et al*., 1996), but overall the number of molecules on the cell surface would be averaged out by the multigene family (Kaufman and Salomonsen, 1997).

Of the many mechanisms by which this expression level could affect resistance to MD, the involvement of NK cells is most attractive (Kaufman and Salomonsen, 1997). Several reports indicate that NK cell activity could be an important facet of resistance to MD in chickens (Sharma, 1981; Garcia-Camacho *et al*., 2003). There are three genetic loci in chickens known to contain NK receptor-like genes. The region syntenic to the NK complex (NKC) in chickens is located on chromosome 1, contains two lectin-like genes (in the draft chicken genome sequence; Rogers *et al*., 2005) and was found confer resistance to MD (Bumstead, 1998). In mouse, this region along with the MHC determines resistance to another herpesvirus, the murine cytomegalovirus (Scalzo *et al*., 1992). The second region is syntenic to the leukocyte receptor complex (LRC), located on a non-MHC microchromosome, and contains a large array of Ig genes, similar to human KIR genes (Viertlboeck *et al*., 2005; Laun *et al*., 2006). Interactions between the KIR and MHC in human disease are well documented (Parham, 2005; Rajagopalan and Long, 2005; Carrington and Martin 2006), including resistance to yet another herpesvirus, Epstein–Barr virus (Butsch Kovacic *et al*., 2005). The third region with apparent NK receptor(s) is the classical MHC of chickens, which was unexpected since there are no lectin-like NK cell receptors found in the MHC of mammals. Of the two such genes, B-NK is most closely related to NKR-P1 in mammals, is transcribed in an NK cell line but not T, B or macrophage lines (Kaufman *et al*., 1999a, b; Rogers *et al*., 2005), and is both highly polymorphic and moderately diverse (Rogers, 2002). One attractive hypothesis is that this lectin-like NK cell receptor gene works specifically (or best) with the dominantly-expressed class I gene from the same MHC haplotype, with some of the haplotype-specific interactions being better than others for resistance to MDV (and likely worse for resistance to certain other pathogens). Examples of such haplotype-specific co-evolution are discussed below. The reports that MDV infection downregulates expression of chicken class I molecules (Hunt *et al*., 2001; Levy *et al*., 2003) could be consistent with this hypothesis if there is a hierarchy of interaction between the relevant MDV-encoded molecule and the class I molecules of different haplotypes.

THE SINGLE DOMINANTLY-EXPRESSED CLASS I MOLECULE IS DUE TO CO-EVOLUTION WITH TAP AND TAPASIN

The picture so far seems clear but counter-intuitive. The chicken MHC has two class I genes and two class II B genes, but it only uses one of each, which means that there can be individuals who are not resistant to a pathogen or responsive to a vaccine because the single dominantly-expressed class I molecule does not find and present a peptide that confers protection. If the chicken simply used all the genes, then a chicken MHC haplotype would be more like mammals, in which the multigene family ensures that most MHC haplotypes confer more-or-less protection to most pathogens. However, chickens stubbornly opt for single dominantly-expressed class I and II genes, with the four independent events "down-grading" different BF1

alleles suggesting that there has been repeated selection pressure against peptide presentation by this poorly-expressed gene.

The reason for the single dominantly-expressed class I gene is rooted in the fact that there is little recombination across the classical MHC of the chicken (which likely is due to the compact and simple nature without much repetitive sequence). There is good evidence for recombination across the classical chicken MHC being rare. In several studies, thousands of matings were examined by serology (and by DNA typing) for recombination between the class I and II genes, without a single recombinant being observed (Hala *et al*., 1979, 1988; Koch *et al*., 1983; Skjødt *et al*., 1985). Also, only one obvious recombinant MHC haplotype between the BF2 and BLB2 genes has been described (Simonsen *et al*., 1982; Jacob *et al*., 2000; Wallny *et al*., 2006), although others may be interpreted in this way (based on sharing alleles at the BF1 locus). Moreover, the similarity of phylogenetic trees for BF1 and BLB1 genes suggest stable haplotypes over evolutionary time (Shaw *et al*., 2007).

The lack of frequent recombination means that groups of genes can co-evolve, allowing the encoded proteins to work together specifically within a haplotype. This idea of co-evolution grew from work on the assembly of class II α and β chains in mice (Germain *et al*., 1985). Polymorphic A and B genes that encode interacting protein chains are virtually inseparable by recombination so that the appropriate α and β chains fit together, whereas A and B genes that are separated by relatively frequent recombination have one gene evolving to an "average best-fit" for the many possible alleles of the other gene.

This work has been extended to the rat MHC (Joly *et al*., 1998), in which class I genes are relatively close to the TAP genes (unlike human and mouse). Rat TAP2 has two allelic lineages, determining TAP proteins which translocate peptides with some specificity at one position (C-terminal amino acid being hydrophobic only versus any residue), and the closely linked class I genes encode class I molecules with the same requirements. Recombinants with the inappropriate combinations lead to low levels of cell surface expression of class I molecules. In humans and mice, the TAP and tapasin genes are located far away from the class I genes which they serve, and are separated by significant levels of recombination (Carrington, 1999; MHC Sequencing Consortium, 1999). As might be expected, TAP and tapasin genes in humans and mice have little sequence polymorphism and no functional polymorphism, having evolved to function with all classical class I loci and alleles.

In comparison to mammals, chicken TAP1, TAP2 and tapasin genes have high allelic polymorphism and moderate sequence diversity, consistent with co-evolution of tightly linked genes. Moreover, the phylogenetic trees of TAP2 and tapasin sequences are both very similar to the dominantly-expressed class I gene BF2 (and not to other genes, such as the apparently neutrally evolving genes BF1 and BLB1, or the highly selected dominantly-expressed class II gene BLB2), as would be expected if the proteins encoded by BF2, TAP2 and tapasin needed to evolve to work together (Kaufman *et al*., 1999a; Walker, 2000; Walker *et al*., 2005; van Hateren, A., Williams, A., Jacob, J., Elliot, T. and Kaufman, J., in preparation).

There is good functional evidence that these genes have evolved to work together within haplotypes (Walker, B., van Hateren, A., Hunt, L., Johnston, F., Marston, D., Shaw, I., Goebel, T., Lehner, P. and Kaufman, J., in preparation). The peptide translocation specificities of three haplotypes (B4, B15 and B21) have been examined, showing that they each pump a different set of peptides specified in at least three positions which match important residues (including the anchor residues) of the peptides found bound to the dominantly-expressed class I molecule. Indeed, the class I molecules are capable of binding a much wider range of peptides than are found bound to the class I molecules on the cell surface and, as illustrated for the B15 haplotype, the TAPs restrict the peptides available to the class I molecules. There are several interesting consequences of this TAP restriction that need to be investigated, but it is clear that a particular class I molecule may receive additional peptides in a heterozygote compared to a homozygote.

The ability of tapasin molecules to function in a haplotype-specific fashion was examined using the "natural recombinant" B19var1, which has a class I molecule and peptide translocation specificity like B15, but a tapasin molecule identical to B12 (van Hateren, A., Williams, A., Jacob, J., Elliot, T. and Kaufman, J., in preparation). Transfection of B15 cells showed that

the dominantly-expressed class I molecule BF2*1902 did not mature as completely as the BF2*1501, as expected if it did not associate well with B15 tapasin. BF2*1902 differs from BF2*1501 in only eight residues, two of which are located in regions thought to be involved in tapasin interaction. These two surface residues in BF2*1902 are the same as in BF2*1201, as though they evolved to allow association with the B12 tapasin that is found in the B19var1 haplotype. Exchange of these residues increased maturation of BF2*1902 and decreased maturation of BF2*1501 in B15 cells, as expected if they are critical for tapasin interactions.

The co-evolution of the class I genes with the antigen-processing genes is important as an explanation for the single dominantly-expressed class I gene, as can be illustrated by the B4 haplotype (Fig. 8.4; Kaufman *et al*., 1999a). The dominantly-expressed class I molecule BF2*0401 has a peptide motif with three negative charges (aspartic acid or glutamic acid at positions 2 and 5, and glutamic acid only at position 8), and there are arginines located appropriately in the peptide-binding site to bind such peptides, while the binding site of the poorly-expressed BF1*0401 does not have appropriate residues to bind such peptides. The TAP1 sequence from the B4 haplotype has three positive charges where other haplotypes have negative charges, and indeed the translocation specificity of B4 cells specifies aspartic acid or glutamic acid at positions 2 and 5, and glutamic acid only at position 8. The important point is that the peptide translocation specificity of the TAP has converged with the peptide-binding specificity of the dominantly-expressed class I molecule, so that few peptides are pumped which can bind the poorly-expressed class I molecule. By inspection of the class I sequences, most haplotypes have very different peptide motifs for the BF1 and BF2 molecules, although the poorly-expressed molecule of the B19var1 haplotype may bind similar peptides as the dominantly-expressed molecule. Thus, it does not really matter how many other class I genes are present, they will not be very important for antigen presentation if their peptide-binding specificities do not match the peptide translocation specificity.

Other genes within the chicken MHC may also be co-evolving. In particular, it would be satisfying to show co-evolution is responsible for only one of the two class II B genes being highly expressed at the RNA level. In fact, there is high polymorphism and limited diversity in all three DM genes, but there are only a few similarities in the phylogenetic trees between either of the class II B genes and any of the DM genes (Atkinson *et al*., 2001). Another appealing possibility discussed above is that the B-NK gene, which encodes a polymorphic lectin-like NK receptor-like protein (Rogers, 2002; Rogers *et al*., 2005), co-evolves with the dominantly-expressed class I gene.

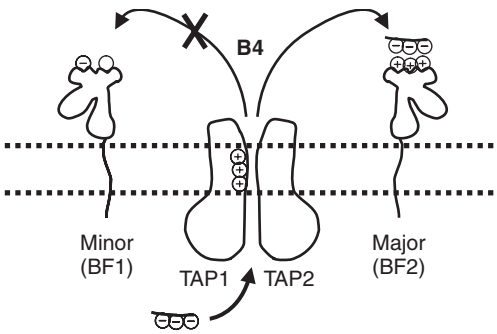

FIGURE 8.4 Only peptides with three negatively charged residues (at positions 2, 5 and 8) are transported by the TAP from the B4 haplotype, presumably because of the three positively charged residues of the TAP1 molecule of the B4 haplotype. The peptides then bind to the dominantly-expressed (major, BF2) class I molecule which has three positively-charged peptide-binding residues and not to the poorly-expressed (minor, BF1) class I molecule which does not have such residues (adapted from Kaufman *et al*., 1999b).

THE CHICKEN MHC GIVES INSIGHTS INTO THE PRIMORDIAL MHC AND THE SUBSEQUENT EVOLUTION OF THE MHC

The chicken MHC has many features that are unlike those of typical mammals, some of which may provide profound insight into the evolutionary history of the MHC. This is particularly clear now for the organization of those genes that are present, the lack of some expected genes and the presence of some unexpected genes, as discussed below.

First, as discussed above, the existence of a single dominantly-expressed class I molecule in chickens is due to co-evolution with the closely linked and therefore polymorphic TAP and tapasin genes, whereas the multigene family of highly polymorphic class I molecules in humans and most mammals is a consequence of the much larger distance to the TAP, tapasin and LMP genes, which evolve to express monomorphic average best-fit molecules. Looking at the available information for other non-mammalian vertebrates, it appears that most non-mammalian MHCs have at least some of the salient features of the chicken MHC rather than the MHCs of typical mammals (Kaufman, 1999). In the frog *Xenopus* (Nonaka *et al*., 1997; Ohta *et al*., 2006), there is only a single classical class I gene which is located near the TAP and one LMP gene (although recombinants can be detected between them). Moreover, the class I and II regions are next to each other, with the class III region genes located outside. In many bony fish (Michalova *et al*., 2000; Sato *et al*., 2000; Stet *et al*., 2003), the classical class I gene(s) is located in a genetic locus with TAP, tapasin and LMP genes completely unlinked to class II and (at least most) class III genes. In the Atlantic salmon (Grimholt *et al*., 2002, 2003), there is only one well-expressed class I gene, at least one closely-linked TAP gene is polymorphic and there is evidence for strong association with resistance to pathogens. In the cartilaginous fish, the shark *Triakis scyllia* express two classical class I genes of which one has fewer alleles or is not expressed in some individuals (Okamura *et al*., 1997). In the nurse shark *Ginglymostoma cirratum*, the single classical class I gene is located close to the TAP and LMP genes (Ohta *et al*., 2002).

In birds there has been more controversy about the organization, particularly focusing on whether all birds have a "minimal essential MHC". The overall organization of the quail MHC is similar to the chicken MHC, but it appears that at both ends there has been repeated tandem duplication of groups of genes, leading to multiple class I, class II B, lectin-like and BG genes. Moreover, there seem to be at least four expressed class I genes, although only two appear to be classical and only one appears to be well-expressed (Shiina *et al*., 1999a, b, 2004, 2006). The duck has five classical class I genes arranged in tandem next to the TAP genes, but like chickens the TAP genes are polymorphic and only one class I gene is expressed at a high level (Mesa *et al*., 2004; Moon *et al*., 2005). The situation is less clear for the passerine birds, with many reports of multiple class I and II genes, of which several are expressed (Westerdahl *et al*., 1999, 2000, 2004, 2005; Freeman-Gallant *et al*., 2002; Ekblom *et al*., 2003; Bonneaud *et al*., 2004), so it is possible that galliform and passerine birds are different. However, it is not clear in these studies whether all of the genes reported are classical genes in the classical MHC, or whether they are all expressed at high levels. Given that even the BF1 gene in chickens has some expression at the RNA level, some allelic polymorphism and some sequence diversity, it is possible that the presence of a dominantly-expressed class I (and class II B) gene has been overlooked.

Thus, many if not most non-mammalian vertebrates have the important features discovered for the chicken MHC: polymorphic TAP (and tapasin and LMP) genes near to the classical class I gene(s), of which only one is expressed at a high level. So, why are mammals different? The easiest explanation is that in the lineage leading to mammals, there was an inversion which brought the class III region in between the class I and II regions, but with the endpoints such that the TAP, tapasin and LMP genes were left next to the class II region rather than accompanying the class I genes to their new location. Recently, the sequence of an MHC from a marsupial, the opossum, shows an organization like non-mammalian vertebrates and therefore indicates that this rearrangement took place in the lineage leading to placental mammals (Miska *et al*., 2002; Belov *et al*., 2006). It is interesting that secondary rearrangements in mammals

may recreate aspects of the situation in non-mammalian vertebrates, such as in rats where some level of co-evolution between class I and TAP genes was first reported (Joly *et al*., 1998).

The Rfp-Y region on the other side of the NOR of the chicken MHC microchromosome contains a variable number of non-classical class I genes, of which at least one is expressed, along with lectin-like genes and apparently non-classical class II B genes (Briles *et al*., 1993; Miller *et al*., 1994a, b, 1996; Afanassieff *et al*., 2001; Rogers *et al*., 2003). A large number of non-classical class I genes are found on one end of the MHC of rats and mice (Gunther and Walter, 2000; Kumanovics *et al*., 2003; Hurt *et al*., 2004). Similar to chickens, a locus with multiple non-classical class I genes is found on the same chromosome as the classical *Xenopus* MHC (Flajnik *et al*., 1993; Courtet *et al*., 2001). Thus, the arrangement of a classical MHC with a nearby non-classical locus could be an ancestral situation (as previously suggested, Kaufman *et al*., 1999a).

The second point arises from the fact that there are many genes found in the mammalian MHC that are missing from the chicken MHC, at least as currently understood. The fates of many of these genes, including the inducible proteasome components (LMP) and TNF cytokines, are unknown, given the poor assembly of the MHC microchromosome (number 16). The class II A gene is located 5 cM away from the partner B gene BF/BL region, one of the few examples of class II A and B genes not being found as tight pairs (Salomonsen *et al*., 2003). The difficulty of cloning the gene and the presence of sub-telomeric repeats (Salomonsen *et al*., 2000) suggest that the class II A gene found its way into a complicated area, perhaps near the telomere or one of the large regions of sub-telomeric repeats.

The complement components C4, C2 and factor B (fB) are found in the MHC of mammals and many non-mammalian vertebrates (MHC Sequencing Consortium, 1999; Nonaka and Kimura, 2006). C2 and fB, which encode serine proteases, are found next to each other in the class III region of the mammalian MHC, as though they are the product of a recent duplication. C4 has two isotypes in the human MHC, encoding C4A and C4B with slightly different functional activities. The C4 and C2/fB genes are found in the MHC of the clawed toad *Xenopu*s, the bony fish *Fugu* and the shark *Triakis* (although they are dispersed in the genome of some other bony fish such as zebrafish). In chickens, the fB gene is found near the Rfp-Y locus, on the same chromosome but unlinked to the classical MHC in the B locus, whereas C2 is located on a different microchromosome (Vitved, L., Marston, D., Bloom, S., Bumstead, N., Croojimans, R., Goodchild, M., Holmskov, U., Koch, C., Rogers, S., Salomonsen, J., Teisner, B., Skjødt, K. and Kaufman, J., in preparation). One chicken C4 gene is found on the edge of the BF/BL region, while another is found on the largest macrochromosome (Skjødt, K., Marston, D., Dodds, A., Bumstead, N., Croojimans, R., Goodchild, M., Powell, T., Rogers, S. and Kaufman, J., in preparation). Interestingly, the MHC-encoded C4 gene is most closely related to the C4 genes of amniotes (and has the residues characteristic of C4A), while the other is most closely related to the C4 genes of fish (and has the residues characteristic of C4B). Inspection of the regions containing the C2 and the non-MHC C4 genes does not identify other MHC-like genes, so these seem to have dispersed as single genes (rather than in paralogous regions, as discussed below), apparently like the situation in some bony fish.

There are a large number of butyrophilin genes in the human MHC (Stammers *et al*., 2000; Rhodes *et al*., 2001), but thus far none at all in the chicken MHC (although at least one has been identified elsewhere, as the receptor for subtype C avian leukosis viruses; Elleder *et al*., 2005). It seems most likely that these MHC-encoded butyrophilin genes have no orthologues in the chicken, but are replaced by BG genes, of which at least 15 have been cloned in one haplotype, perhaps associating with the gene products of the single 30.2 genes found nearby (Kaufman *et al*., 1991, unpublished observations). Also, TRIM-B30.2 genes have been identified in chickens, some of which are found in the human MHC, while others along with a guanine nucleotide-binding protein gene have been dispersed in mammals (Guillemot *et al*., 1989; Ruby *et al*., 2005). Thus, the overall conclusion is that genes missing from the chicken MHC have been dispersed for reasons and by mechanisms so far unknown, with different genes dispersed in the lineage leading to mammals.

Third, there are several genes present in or near the chicken MHC that are not found in the mammalian MHC. In the BF/BL region, there are two genes in opposite transcriptional orientation

encoding type II membrane proteins with extracellular C-type lectin-like domains (Kaufman *et al*., 1999a, b; Rogers *et al*., 2005). The B-NK gene has six exons, the RNA is present in NK but not T, B or macrophage lines, and the protein has both an extracellular stalk and an immuno-tyrosine inhibitory motif (ITIM). The Blec gene has five exons, the RNA is rapidly upregulated after stimulation, and the protein has no stalk and no ITIM. This pair of genes is similar to gene pairs in the NKC of humans and mice, each composed of an NK cell receptor-like gene and an early activation antigen gene, such as human NKR-P1 and LLT1, human CD69 and KLRF1, and mouse NKR-P1 and Clr. In contrast, in the region of the chicken genome syntenic to the NKC, there are only two lectin-like genes, one most closely related to CD94/NKG2D and the other most closely related to CD69, which are located some 40 Mbp apart (Chiang *et al*., 2007).

More recently, two CD1 genes were identified around 50 kbp from the dominantly-expressed class I gene (Maruoka *et al*., 2005; Miller *et al*., 2005; Salomonsen *et al*., 2005). In mammals, CD1 genes are found on a non-MHC chromosome, for instance chromosome 1 in humans, along with many other genes homologous to those found in the MHC. However, no class I-like genes were found in the region of the chicken genome apparently syntenic to the CD1 region of humans. The existence of CD1 genes in chickens indicates that the lipid presentation system of CD1 molecules dates back at least to the ancestors of mammals and birds, at least 300 million years ago, but the presence of CD1 genes in the chicken MHC suggests that they arose much earlier.

In humans, four so-called MHC-paralogous regions have been identified on chromosomes 1 (the CD1 region), 6 (the MHC), 9 and 19, each containing an array of some genes like those in the MHC (Kasahara *et al*., 1996; Kasahara, 1999). For instance, the gene for the complement component C4 is found in the MHC, while the homologous C3 and C5 genes are found in the paralogous regions on human chromosomes 3 and 9, respectively. These regions are thought to have arisen during two rounds of genome-wide duplication before the emergence of bony and cartilaginous fish around 600 million years ago. Based on the presence of some MHC-like genes, the NKC is thought to represent a fifth MHC-paralogous region (for instance, containing the α_2-macroglobulin gene homologous to C3, C4 and C5), probably broken off from one of the other paralogous regions.

The presence of both the two lectin-like membrane protein genes and the two CD1 genes in the chicken MHC is most easily understood in terms of these paralogous regions (Fig. 8.5), with both sets of genes present in the primordial MHC and then duplicated twice to be present in all paralogous regions. In the lineage leading to birds, these genes were retained in the MHC and silenced in the paralogous regions. In the lineage leading to mammals, the CD1 genes were retained in the CD1 region but lost in the MHC and other paralogous regions, while the lectin-like genes were retained in the NKC but lost in the MHC and other paralogous regions (Maruoka *et al*., 2005; Rogers *et al*., 2005; Salomonsen *et al*., 2005). This idea (but in reverse) may also explain the presence of Ig-like NK cell receptor genes in the human MHC rather than in the LRC (Moretta *et al*., 2001).

It has long been a mystery why some genes involved in antigen processing and presentation, such as those encoding LMP, TAP and tapasin, are present in the MHC. These genes are unrelated in structure to each other and to MHC class I and II molecules, and their ancestors must have had different functions. One possible explanation for this mystery is that the same kind of co-evolution that is occurring for alleles of class I, TAP and tapasin genes in chickens must have happened during the assembly of the primordial MHC. In this view, the ancestor of the LMP genes had to evolve to cleave peptides of a size that might bind class I molecules, the ancestors of the TAP genes had to evolve to pump peptides that might bind class I molecules, and the ancestor of the tapasin gene had to evolve to bind TAP and class I molecules and to function as a class I chaperone and peptide editor. Being genetically closely-linked was important to evolve efficiently from disparate ancestral genes to a smoothly functioning team of genes, much like the evolution of metabolic pathways in the most primitive organisms. Following this line of reasoning, it is likely the primordial MHC originally contained most of the genes involved in antigen presentation and recognition, including NK cell receptor genes and T cell receptor genes. This hypothesis may explain the presence of a gene with similarities to T cell receptors located in the MHC of the clawed toad *Xenopus* (Ohta *et al*., 2006).

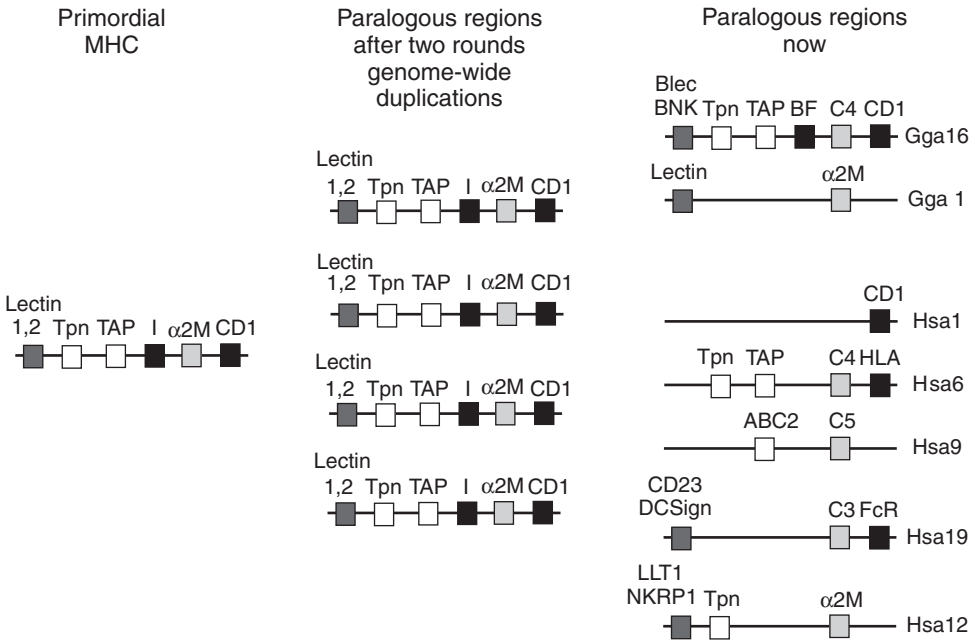

FIGURE 8.5 Genes present in the primordial MHC were multiplied by two rounds of genome-wide duplication into four paralogous regions on different chromosomes. Subsequent differential silencing, deletion and degradation led to different groups of genes to be present together in the lineages leading to mammals and birds. Gga 1 and 16 are chicken chromosomes; Hsa1, 6, 9, 12 and 19 are human chromosomes. CD23, DCSign, LLT1, NKRP1, BNK and lectin 1, 2 all represent members of the lectin-like NK receptor-like family of genes. HLA, CD1, FcR, BF and I all represent members of the class I family of genes. C3, C4, C5 and α2M all represent members of the α_2-macroglobulin family of genes. Tpn and TAP represent tapasin and transporters associated with antigen presentation, respectively. Many genes are not represented for reasons of simplicity (adapted from those in Rogers *et al.*, 2005; Salomonsen *et al.*, 2005).

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9 AVIAN ANTIGEN PRESENTING CELLS

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INTRODUCTION

Antigen Presentation

Antigen presentation is the mechanism by which the antigenic environment is sampled and information imparted to the effector arms of the adaptive immune system, B and T lymphocytes. Depending on the precise context, antigen presentation can result in either activation or tolerization of lymphocytes, respective examples being the response to a pathogen challenge or tolerance to self-antigen. An antigen is subjected to either endogenous or exogenous processing and the resulting peptides are expressed on the surface of the antigen presenting cell (APC) bound to either major histocompatibility complex (MHC) class I or II molecules (see Chapter 8). The type of MHC molecule involved in the presentation not only reflects the source of the antigen, but also has the primary role in determining the ensuing immune response to it.

Almost all cells express class I heterodimer molecules on their outer surface and use the specialized MHC peptide binding cleft to express peptides derived from endogenous antigens, sampled from the cytoplasm or nucleus. The avian MHC region and the proteins it encodes are described in detail in Chapter 8. Endogenous processing degrades the host's own proteins, tumour antigens if a cell has been neoplastically transformed or antigens derived from pathogens, which replicate within the cytoplasm or nucleus. These proteins are degraded to peptides by the proteasome, and the peptides are translocated to the endoplasmic reticulum where they become bound to MHC class I molecules. The peptide stabilizes the MHC molecular complex which is transported to the cell surface via the Golgi apparatus. If an MHC molecule bearing a peptide derived from a foreign or transformed antigen is expressed on the cell surface, the antigen–MHC complex is recognized by a T cell receptor (TCR) on a $CD8^+$ cell. The MHC/TCR interaction induces the $CD8^+$ cell to proliferate and become armed antigen-specific cytotoxic

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T lymphocytes (CTL). Activated CTL are able to kill other cells presenting the same antigen on their surface in the context of MHC class I. In this way, virally-infected or transformed cells are killed, thereby limiting their further replication or spread of the pathogen.

Exogenous antigen can be phagocytosed by specialized APC such as macrophages, or taken up by dendritic cells (DC) and B cells by receptor-mediated absorptive endocytosis, or by DC through macropinocytosis. These exogenous antigens reside within endosomes. Also included here are antigens derived from pathogens, such as *Salmonella*, which invade APC and reside within intracellular vesicles. The endosome fuses with a lysosome-containing acid proteases to form a phagolysosome and this results in the digestion of the exogenous antigens into antigenic peptides. Peptide-containing phagolysosomes fuse with other vesicles which contain the α and β chains of the MHC class II molecule, in the process replacing a stabilizing invariant chain, so that the antigen peptide can be transported to, and presented on, the surface of the cell in the context of the MHC class II molecule. DC are able to place class II MHC–antigen complexes on their surfaces at far higher densities than is achievable by macrophages. DC achieve this by means of the production of subcellular compartments rich in MHC molecules which fuse with the antigen-rich endocytotic vesicles. Antigen–MHC complexes are recognized on the cell surface by the TCR of $CD4^+$ T cells, which then activate other immune effector cells. B cells presenting antigen using MHC class II molecules are induced to secrete antigen-specific antibody by interaction with activated $CD4^+$ Th2 cells of the same specificity. Macrophages harbouring intravesicular bacteria or parasites can be activated and induced to kill them by interaction with Th1 cells. DC also have the ability to load peptides generated in the endosomal pathway onto MHC class I molecules. Although the molecular mechanisms that facilitate this are not fully elucidated, the resulting phenomenon, called cross-priming, ensures that viral infections are able to generate both CTL and humoral responses, irrespective of their site of replication. Co-stimulation, discussed in Chapter 4 in the context of B cells, is essential for the generation of immune response. T cells recognizing antigen on the surface of a DC – in the context of the MHC molecule and co-stimulation – are induced into proliferation and differentiation into effector T cells. T cells recognizing the antigen–MHC complex in the absence of a "second signal" become anergic, even in the face of further stimulation by DC.

Dendritic Cells

Since their identification over 30 years ago, DC, the most potent APC that are able to stimulate naïve T cells in antigen-specific immune response, have been fundamental to the study of immune responses in mammalian species (Steinman *et al.*, 1975). The existence of a specialist APC in birds has long been speculated but until recently evidence was mostly circumstantial. Of the three types of mammalian professional APC: B cells, macrophages and DC, the first one can be excluded as being necessary for antigen presentation since bursectomized birds, lacking in B cells, can mount normal T cell responses (Beal *et al.*, 2006). The possibility remains that avian macrophages may be sufficient to mount normal immune responses. However, it is only with (1) the recent advent of recombinant cytokines, (2) the development of isolation protocols and (3) the generation of DC-relevant antibodies that studies on avian DC as a distinct population have been possible. Although only now we are attaining an understanding of avian DC biology, it is already apparent that many of the characteristics of mammalian DC – a prerequisite to their unique function – are replicated in whole or in part in birds. Whilst much of the molecular machinery which allows DC to present antigen has been described for the chicken (see Chapter 8), this is not of itself sufficient to define an immune surveillance system. The acts of antigen uptake, processing and presentation place different requirements on the APC, resulting in a change of state from the so-called immature phenotype, which is efficient at antigen capture, to the "mature" phenotype that is optimal for antigen presentation. Furthermore, antigen capture may take place at a site which is anatomically distant from where antigen presentation to lymphocytes occurs; and DC must therefore also have, or acquire, the ability to selectively migrate. These issues are discussed later.

Macrophages

Macrophages represent a heterogeneous group of cells which are found throughout the body in both vertebrates and invertebrates (Gordon and Taylor, 2005). The term macrophage was introduced in 1884 by Ilya Metchnikoff to describe leukocytes which are capable of ingesting and destroying foreign substances including micro-organisms (Karnovsky, 1981). Since then it has become clear that these cells play a fundamental role in tissue homeostasis, innate and acquired immunity, and inflammation and immunopathology (Gordon, 2003). To accomplish this multiplicity of tasks, macrophages detect environmental signals through specific receptors, phagocytose apoptotic and necrotic cells as well as invading micro-organisms, and respond to these stimuli by the secretion of signalling and effector molecules. Much progress has been made in the field of macrophage biology in recent years by definition of the structure and function of their molecules in some mammalian species (Taylor *et al*., 2005). By comparison, knowledge on avian macrophages is very limited and the number of macrophage-specific tools is restricted. Here, we will give an overview of avian macrophage biology.

DEVELOPMENT OF MYELOID CELLS

Resident tissue macrophages, as well as DC and osteoclasts, develop from circulating monocytes originating from myeloblast progenitors in the bone marrow (Shepard and Zon, 2000). These progenitor cells can also give rise to granulocytes with the lineage decision being regulated by haematopoietic growth factors and the interaction of transcription factors (Sieweke and Graf, 1998; Kelly *et al*., 2000). Monocytes differentiate in the bone marrow in response to granulocyte/macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF), amongst others (Barreda *et al*., 2004). These growth factors, or cytokines, are released into the peripheral blood whence they enter the tissues to develop into resident macrophages under the influence of tissue-specific signals. In the chicken, myelopoiesis has been studied with the help of avian retroviruses which express the v-*myc* oncogene (e.g. MC29) and induce myelocytomatosis *in vivo* or the v-*myb* oncogene (e.g. E26 or avian myeloblastosis virus) which induces myeloblastosis. These and other avian leukosis viruses have also been used to investigate haematopoiesis in the chicken system. A detailed description of this system can be found in McNagny and Graf (1996).

Chicken myeloid cells transformed by v-*myc* or v-*myb* require a growth factor, secreted by activated macrophages and termed chicken myelomonocytic growth factor (cMGF; Leutz *et al*., 1984), for proliferation. cMGF was the first chicken cytokine to be cloned and characterized at the molecular and functional level and shown to be related to mammalian G-CSF and interleukin (IL)-6 (Leutz *et al*., 1989). Interestingly, no mammalian homologue to cMGF has been described so far and an initial analysis of the chicken genome has failed to identify this cytokine in the published sequence (Kaiser *et al*., 2005). Binding of cMGF to myeloid progenitor cells causes rapid STAT5 phosphorylation followed by proliferation and further differentiation (Woldman *et al*., 1997) in *in vitro* systems. *In vivo*, overexpression with a recombinant fowlpox virus significantly increased the number of circulating monocytes and their activation status (York *et al*., 1996) confirming the functional relevance of this cytokine. Recently, chicken GM-CSF has been cloned, shown to be functionally active (Avery *et al*., 2004), and has been used for the *in vitro* differentiation of myelomonocytic stem cells into macrophages and DC (Z. Wu and L. Chappell, personal communication). In addition to STAT5, several other transcription factors have been implicated in monocytic differentiation (Kowenz-Leutz *et al*., 1997) but most of them are not strictly lineage specific, with the possible exception of MafB (Kelly *et al*., 2000). Using the v-*myb*-transformed chicken monoblastic cell line BM2, Benes *et al*. (2006) identified the intermediate filament protein vimentin as another regulatory factor in monocyte/macrophage differentiation in the chicken.

Monocytes constitute approximately 5–10% of peripheral blood leukocytes but this number may vary considerably between different chicken lines. In mammals, it was shown that these cells do not represent a homogeneous population. Rather, several monocyte subsets can be discriminated using cell surface markers; they seem to reflect developmental stages with distinct physiological roles (Gordon and Taylor, 2005). These differences become even more obvious as monocytes differentiate into resident macrophages and DC in the tissues. The lack of markers in the avian system has prevented the definition of monocyte subsets but macrophage heterogeneity has been described histologically (Jeurissen *et al.*, 2004; Igyarto *et al.*, 2006).

Embryonic macrophages may not follow the same pathway, described above for postnatal monocyte/macrophage development, and may therefore represent an independent lineage. Studies with yolk sac chimaeras (see Chapter 3) have shown that primitive haematopoietic cells arise from yolk sac mesoderm during early development, whereas definitive blood cells are derived from intra-embryonic precursors (Dieterlen-Lierve, 1984). In the yolk sac of embryos incubated for 2.5–4.5 days, cells with macrophage-like morphology have been found and these increase in number during development, showing phagocytic activity in areas of cell death (Cuadros *et al.*, 1992, 1993). It has been suggested that these cells play a crucial role in the removal of apoptotic cells during development and are homologues of the mammalian foetal macrophages (Shepard and Zon, 2000).

SOURCES FOR AVIAN MACROPHAGES AND DC

Several sources for avian macrophages have been described, the most important ones being peritoneal exudate cells and monocyte-derived macrophages. In mice and rats, macrophages are constitutively present on the serosal membranes of the peritoneal cavity and peritoneal lavage yields significant numbers of non-activated resting macrophages (Conrad, 1981). In contrast, these cells are essentially absent in the chicken and macrophages have to be recruited from the circulation into the body cavity by inflammatory stimuli prior to the lavage procedure (Rose and Hesketh, 1974; Sabet *et al.*, 1977). Comparison of different stimulation protocols revealed that a single injection of a 3% Sephadex G-40 suspension in saline gives optimal results and primarily attracts cells with macrophage morphology. Repeated Sephadex injections increase the number of lymphocytes in the samples, whereas the injection of starch primarily elicits heterophils (Trembicki *et al.*, 1984). Sephadex-elicited cells are strongly adherent to tissue culture plastic, show macrophage morphology and characteristic macrophage activities such as phagocytosis and bacterial lysis (Dietert *et al.*, 1991; Myszewski and Stern, 1991). Comparable results were obtained for turkeys (Qureshi *et al.*, 2000) and quails (Trembicki *et al.*, 1984) suggesting that this procedure can be applied to most avian species. It should be noted that Sephadex-elicited macrophages are functionally activated cells (Qureshi *et al.*, 1986) and may respond differently to cells obtained from other sources (Plate 9.1).

As an alternative technique, mononuclear cells have been isolated from different lymphoid organs, bone marrow or blood by adherence to glass or tissue culture plastic surfaces. Carrell and Ebeling (1922) were the first to describe the generation of pure macrophage cultures from chicken blood, a system later used to demonstrate the formation of epitheloid and giant cells (Lewis, 1925) and the differentiation of monocytes to macrophages (Sutton and Weiss, 1966). The adherence of chicken monocytes is slow and requires one to several days of culture rather than hours, as routinely used for the selection of human monocytes and macrophages (Edelson and Cohn, 1976). Importantly, short-term cultures of peripheral blood mononuclear cells (PBMC) separated by density-gradient centrifugation give rise to nearly pure thrombocyte preparations after removal of non-adherent cells (Grecchi *et al.*, 1980). The nucleated avian thrombocytes attach to both glass and plastic surfaces within 30 min (Janzarik *et al.*, 1980) but die within 48–72 h or are actively phagocytosed by the adherent macrophages in the cultures (Sutton and Weiss, 1966). Thus, a protocol has been established in which monocyte-derived macrophages can be selected from peripheral blood by adherence to glass or plastic for at least 48 h (Peck *et al.*, 1982). Since these cells adhere firmly non-adherent and dead cells can

easily be removed by vigorously washing the culture dishes. In contrast to Sephadex-elicited macrophages, these cells display a non-activated phenotype with comparatively weak phagocytosis of non-opsonized particles and low-level MHC class II antigen expression (Kaspers *et al*., 1994). These may therefore be a preferential cell source for studies on macrophage differentiation and activation. Resident tissue macrophages have also been isolated for functional studies. These cells are usually selected from cell suspensions obtained by tissue dissociation through adherence, as described for monocyte-derived macrophages (Peck *et al*., 1982; Rossi and Himmelhoch, 1983) (Plate 9.2).

At the 2006 Avian Immunology Research Group Meeting (Paris), several groups reported the generation of avian DC from blood-derived monocyte preparations (M. Kahatri and J. Sharma) and bone marrow cells (Z. Wu) by culture using recombinant chicken GM-CSF and IL-4. We have also reported the extraction of populations of cells from chicken spleen and lung that appear to be functional DC. Based on standard isolation procedures for mammalian DC (Robinson and Stagg, 2001), these putative chicken DC were obtained using adherence and discontinuous density-gradient centrifugation.

AVIAN MYELOID CELL LINES

Myeloid cell lines have been of great value for studies on lineage differentiation and avian macrophage function. As discussed above, myeloblast and macrophage-like cell lines can be obtained by retroviral transformation *in vitro* and *in vivo*. By using the v-*myc*-containing avian myelocytomatosis virus to MC29 Beug *et al*. (1979) developed a macrophage-like cell line initially described as LSCC-HD (MC/MA1), which was subsequently renamed HD11 (Leutz *et al*., 1984; Plate 9.1(f)). In the original report, it was shown that these cells are slightly adherent, express Fc receptors, actively phagocytose bacteria and react with a macrophage-specific antiserum but lack markers of other haematopoietic cell lineages (Beug *et al*., 1979). Since then HD11 cells have been used by numerous investigators to study avian macrophage biology and this has led to improved characterization. A summary of the major phenotypic and functional characteristics of the HD11 cell line is given in Table 9.1.

The BM2 myeloblastoid cell line was established by transformation with avian myelocytomatosis virus containing the v-*myb* oncogene (Moscovici *et al*., 1982). This non-producer cell line displays blastoid morphology and expresses Fc receptors but lacks Fc-receptor-mediated phagocytosis. Upon stimulation with lipopolysaccharide (LPS), BM2 cells acquire a phenotype more closely related to macrophages. Additional treatment of these cells with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) induces terminal differentiation to mature macrophages, an effect not reversed by TPA removal (Symonds *et al*., 1984). BM2 cells have subsequently been used to investigate the differentiation of monocytes and macrophages (Benes *et al*., 2006) and osteoclasts (Solari *et al*., 1996).

The third avian macrophage-like cell line was obtained from a chicken which developed splenomegaly after infection with the JM/102W strain of the Marek's diseases virus. This cell line, called MQ-NCSU, was shown to phagocytose sheep red blood cells (SRBC) and bacteria, to express Fc receptors, high levels of MHC class II antigens and transferrin receptors. In addition, these cells react with the monoclonal antibody (mAb) K1 which is specific for monocytes/macrophages and thrombocytes (Qureshi *et al*., 1990) and display the morphological characteristics of malignantly transformed mononuclear phagocytes. Thus far, the transforming agent has not been described for this cell line. It should however be noted that this line also stains with a polyclonal anti-REV serum (Calnek and Schat, unpublished observations).

Recently, another mononuclear cell line displaying monocyte/macrophage morphology and functional characteristics of macrophages was established from cells growing out of a primary chicken heterophil culture. This cell line, termed HTC, was shown to be positive for Marek's disease virus and avian leukosis virus using PCR analysis and to produce leukosis virus antigen detected by enzyme-linked immunosorbent assay (ELISA) suggesting that the cells were most likely transformed by avian leukosis virus (Rath *et al*., 2003).

TABLE 9.1 Some Phenotypic Properties of the Chicken Macrophage-Like Cell Line HD11 (Beug *et al.*, 1979)

Phenotype		Reference
Cell surface antigens	MHC class I antigen	Xie *et al.* (2003)
	MHC class II antigen	Golemboski *et al.* (1990)
	Transferrin receptor	Golemboski *et al.* (1990)
	IL-15R α chain	Li *et al.* (2001)
	CD40	Tregaskes *et al.* (2005)
	K1 antigen	Kaspers *et al.* (1993)
	K55 antigen	Xie *et al.* (2003)
	KUL01 antigen	Mast *et al.* (1998)
TLR expression*	TLR1/6/10 homologue	Iqbal *et al.* (2005a)
	TLR2 type-1	
	TLR2 type-2	
	TLR3	
	TLR4	
	TLR5	
	TLR7	
Phagocytosis	Fc-receptor-mediated lysis of SRBC and *Salmonella*	Beug *et al.* (1979) Xie *et al.* (2003)
Anti-microbial factors	Oxidative burst	Chadfield and Olsen (2001)
	Nitric oxide production	Sung *et al.* (1991)
	Lysozyme expression	Goethe and Loc (1994)
	Arachidonic acid metabolites	Golemboski *et al.* (1990)
Cytokine expression	cMGF	Leutz *et al.* (1989)
	IL-1β	Smith *et al.* (2005)
	IL-6	Smith *et al.* (2005), Xie *et al.* (2002)
	IL-8*	Smith *et al.* (2005)
	IL-10	Rothwell *et al.* (2004)
	K60*	Smith *et al.* (2005)
	TL1a	Hong *et al.* (2006)
Cell activation by	Polyinositol:cytosine	He *et al.* (2007)
	LPS	Sung *et al.* (1991)
	Flagellin	Iqbal *et al.* (2005b)
	R848	Philbin *et al.* (2005)
	CpG ODN	He and Kogut (2003)
	IFN-γ	Digby and Lowenthal (1995)

Citations do not necessarily refer to the original reports.
*For details on chicken TLR and chemokine nomenclatures, see Chapter 7 and 10, respectively.

Lawson and colleagues (2001) established the turkey macrophage cell line IAH30 by transformation of peripheral blood leukocytes with the avian leukosis virus strain 966 (Lawson *et al.*, 2001). This cell line reacted with mAb against chicken β_2-microglobulin and chicken monocyte/macrophage/thrombocyte marker (recognized by the mAb K1) and secreted nitric oxide (NO) upon interferon (IFN)-γ stimulation but did not express the macrophage marker recognized by the mAb KUL01.

Cell Surface Markers for Avian Myeloid Cells

The first mAb reacting with myeloid cells of the chicken were developed by immunizing mice with avian myelocytomatosis virus- and MC29-transformed chicken myeloblasts and macrophages. Four antibodies were selected that were specific for myeloid cells (Kornfeld *et al.*, 1983). The mAb 51-2 has subsequently been used for the identification of myeloid cells by others (al Moustafa *et al.*, 1994). Using Sephadex-elicited abdominal exudate macrophages as

immunogen, Trembicki *et al.* (1986) established the mAb CMTD-1 and CMTD-2, which react with mature macrophages but not react with blood monocytes or myeloid precursors (Trembicki *et al.*, 1986). These are thought to detect a carbohydrate epitope on inflammatory macrophages (Qureshi *et al.*, 2000). The mAb CVI-ChNL-68.1 specifically reacts with a cytoplasmic antigen of monocytes, macrophages and interdigitating cells in tissue sections but does not stain the cell surface of myeloid cells in suspension (Jeurissen *et al.*, 1988). A more restricted reaction pattern has been found for the mAb CVI-ChNL-74.2, which only recognizes a subpopulation of macrophages in lymphoid tissues (Jeurissen *et al.*, 1992). The mAb K1, selected from a number of antibodies obtained after immunizing mice with chicken spleen cell suspensions, was initially described as monocyte/macrophage specific (Chung and Lillehoj, 1991). Subsequently, it was shown that this antibody reacts with a heterodimeric cell surface protein shared by monocytes/macrophages and thrombocytes (Kaspers *et al.*, 1993). This mAb has been used in flow cytometry and immunohistology to identify monocytes and macrophages (Kaspers *et al.*, 1993; Olah *et al.*, 2002) and to discriminate lymphocytes from thrombocytes in peripheral blood leukocyte preparations obtained by density-gradient centrifugation (Bohls *et al.*, 2006). It should be noted, however, that thrombocytes can be stained more specifically with mAb 11C7 which reacts with the thrombocyte-restricted integrin gpIIb/IIIa, the fibrinogen receptor (Lacoste-Eleaume *et al.*, 1994). Finally, mAb K1 has also been used in other avian species including guinea fowl (Olah *et al.*, 2002) and duck (Kothlow *et al.*, 2005).

The most specific mAb for cells of the mononuclear phagocyte system of the chicken was developed by Mast *et al.* (1998) and designated KUL01. This antibody has proved to be a valuable tool, both for flow cytometry and in immunohistology, in several studies (Van Immerseel *et al.*, 2002; Barrow *et al.*, 2003; Khatri *et al.*, 2005; Tregaskes *et al.*, 2005; Palmquist *et al.*, 2006). In tissue sections, KUL01 stains macrophages and interdigitating cells, as well as cells resembling mammalian epidermal Langerhans cells (Mast *et al.*, 1998).

Despite considerable efforts to develop myeloid cell markers, so far no lineage-specific mAb has been obtained for which the antigen has been defined at the molecular level. Consequently, the expression of the antigens identified by the described monocyte/macrophage markers cannot be correlated with particular functional properties. However, the availability of the chicken genome sequence should help to identify these antigens and to aid the generation of new mAb against lineage-specific antigens expressed by recombinant DNA technology.

While the number of myeloid-specific mAb is limited several antigens have been identified which are simultaneously expressed on monocytes/macrophages and on other leukocytes. A range of mAb was used to demonstrate MHC class II antigen expression on monocytes and macrophages including mAb CIa-1 (Ewert *et al.*, 1984), 21-1A6 (Veromaa *et al.*, 1988), P2M11 (Kaspers *et al.*, 1993) and 2G11 (Lawson *et al.*, 2001). MHC class II antigen expression was shown for myeloid cells of chickens (Ewert *et al.*, 1984), quails and turkeys (Lawson *et al.*, 2001). In addition, monocytes/macrophages were found to be MHC class I positive (Vainio *et al.*, 1983), to express the CD40 (Tregaskes *et al.*, 2005), CD44 (Rath *et al.*, 2003) and CD45 (Chung and Lillehoj, 1991; Rath *et al.*, 2003) antigens and the IL-15 receptor alpha chain (Li *et al.*, 2001). Attempts to demonstrate CD4 expression on chicken monocytes, analogous to its expression on human monocytes, have failed (Mast *et al.*, 1998).

The recent production of an mAb for chicken DEC205 allows the localization of the chicken DC endocytosis receptor (K. Staines, personal communication). In common with mammalian species this is, in itself, insufficient to identify DC. However, it may be used in combination with the markers described above to describe the potential subpopulations of immature DC.

CHARACTERIZATION OF MACROPHAGES AND DC IN TISSUE SECTIONS

Specific mAb for myeloid cells have been used to identify macrophages and, to a more limited extent, DC in their physiological contexts. This has been done using a range of tissues and in a

variety of *in situ* pathological settings, during ontogeny and organ development. The distribution and morphological features of these tissue macrophages has been discussed in Chapter 2.

Chicken Langerhans-like cells were first described as adenosine triphosphate positive and MHC class II^+ (Carrillo-Farga *et al*., 1991; Perez Torres and Millan Aldaco, 1994). More recent work demonstrated that these cells express the CD45 antigen and have a cytoplasm rich in vimentin intermediate filaments (Igyarto *et al*., 2006). These workers also described subpopulations of avian DC and suggested phenotypes based on MHC class II and CD3 expression: $MHCII^+/CD3^-$, $MHCII^-/CD3^-$ or $MHCII^-/CD3^+$, with the first two types representing Langerhans cells. Other workers have described morphological DC in various tissues (Olah and Glick, 1985, 1995; Toro and Olah, 1989).

FUNCTIONAL PROPERTIES OF CHICKEN MACROPHAGES

Macrophages display a series of functional characteristics which allow them to perform their multiple tasks in tissue homeostasis, pathogen recognition and destruction, modulation of innate immune responses and activation of the adaptive immune system.

Macrophage Migration

To develop into resident tissue macrophages under normal physiological conditions or during inflammation, monocytes have to leave the circulation and migrate to an appropriate location in response to chemotactic signals. The molecules and signals involved in this process are largely undefined for avian macrophages, but it may be assumed that they are similar to those described for their murine and human counterparts. Histological studies have, however, demonstrated the adhesion of macrophages to the blood vessel wall and their active migration to sites of infection and inflammation. Dietert and co-workers used Sephadex particles to induce an inflammatory environment in the abdominal cavity of chickens and turkeys and were able to describe the kinetics of monocyte attraction, and their functional maturation to inflammatory macrophages (Trembicki *et al*., 1986; Chu and Dietert, 1988; Golemboski *et al*., 1990). In this system, macrophages are rapidly and selectively recruited to the inflammatory site and make up more than 80% and 95% of the adherent cell population in the abdominal exudate samples after 4 and 72 h, respectively. Since resident macrophages are largely absent on the lung surface (see Chapter 14) and in the abdominal cavity, rapid influx of phagocytes into sites of infection may be essential for the efficient control of pathogens in birds (Toth and Siegel, 1993). Chicken monocyte response to chemotactic signals was first shown by classical migration inhibition assays (Morita *et al*., 1976) and subsequently in *in vitro* experiments with the synthetic signal peptide f-MLP (Qureshi *et al*., 1988). The recent description of the chicken chemokine and chemokine-receptor families (Kaiser *et al*., 2005, and also Chapter 10) should enable more detailed functional studies of monocyte/macrophage migration in the future.

Phagocytosis

Phagocytosis is perhaps the best known and, in evolutionary terms, most conserved function of macrophages that has been used to identify these cells *in situ* and to discriminate them from other leukocytes in mixed cell preparations. Phagocytosis of particles and micro-organisms is mediated by specific cell surface receptors which have been characterized in great detail for mammals (Taylor *et al*., 2005).

In chicken embryos, macrophages with phagocytic activity have been observed as early as embryonic incubation day (EID) 12 in the liver and EID 16 in the spleen (Jeurissen and Janse, 1989). Elicited macrophages can be obtained in day-old chicks and turkey poults (Qureshi *et al*., 2000) demonstrating that this part of the innate immune system is functional at hatching. Cultured monocyte-derived macrophages, Sephadex-elicited macrophages and cells of the macrophage cell lines HD11, MQ-NCSU and HTC spontaneously phagocytose SRBC, fluorescent micro-beads, bacteria or other particulate matter to different degrees (Beug *et al*., 1979;

Chu and Dietert, 1988; Qureshi *et al*., 1990; Rath *et al*., 2003; Plate 9.1(c) and (d)). Although elicited macrophages harvested early after inflammatory stimulation of the abdominal cavity are efficient in phagocytosis, this can be significantly increased if cells are collected at a later time, indicating that macrophages undergo functional maturation in response to inflammatory stimuli (Chu and Dietert, 1988). Interestingly, phagocytosis of non-opsonized particles is restricted to a subpopulation, reflecting the functional heterogeneity of primary cell cultures (Qureshi *et al*., 2000) but also of macrophage cell lines (Qureshi *et al*., 1990; Rath *et al*., 2003). In cultures of elicited macrophages, the number of phagocytic cells can be increased from 50% to more than 90% by opsonization with antibodies. This effect correlates with the expression level of Fc receptors, as analyzed by the SRBC-rosette assay (Qureshi *et al*., 2000).

Phagocytosis by primary macrophages has also been demonstrated with a range of bacterial species, including *Salmonella enterica* (Qureshi *et al*., 1986; Okamura *et al*., 2005), *Escherichia coli* (Harmon and Glisson, 1989; Miller *et al*., 1990), *Campylobacter jejuni* (Myszewski and Stern, 1991) and *Pasteurella multocida* (Harmon *et al*., 1992), and the fungal pathogen *Candida albicans* (Rossi and Turba, 1981). Opsonization clearly increases phagocytotic activity *in vitro* for both bacteria (Myszewski and Stern, 1991) and fungi (Rossi and Turba, 1981; Qureshi *et al*., 1986).

Binding of micro-organisms prior to phagocytosis requires receptor-mediated recognition. Macrophages are equipped with a range of receptor systems such as the scavenger receptors, complement receptors, Fc receptors, C-type lectins and mannose receptors mediating opsonic and non-opsonic recognition (Taylor *et al*., 2005). In the avian system no mAb with specificity for members of these receptor families are so far available, except for a mAb to the DC endocytosis receptor. However, functional studies strongly indicate the presence of some of these molecules on primary avian macrophages and macrophage cell lines. SRBC opsonized with anti-SRBC serum from chickens or quails strongly adhere to cultured macrophages and are more efficiently phagocytosed than non-opsonized erythrocytes (Beug *et al*., 1979; Qureshi *et al*., 1990; Qureshi, 2003). The observation that *C. albicans* adheres to macrophages and is rapidly phagocytosed led to the functional characterization of the chicken mannose receptor and its ultrastructural localization on the cell surface (Rossi and Himmelhoch, 1983).

Detection and uptake of micro-organisms induces the activation of effector mechanisms which, in most cases, lead to pathogen destruction. As already mentioned, during phagocytosis particles are internalized into phagosomes which subsequently fuse with lysosomes to form a phagolysosome. Lysosomes contain a variety of anti-microbial proteins and enzymes, such as acid phosphatase and β-glucuronidase; some of which have also been described for avian macrophages (Fox and Solomon, 1981). Histochemical demonstration of non-specific esterase activity has been used to identify macrophages in tissue sections (Schaefer *et al*., 1985; Jeurissen *et al*., 1992; Schnegg *et al*., 1994; Mast *et al*., 1998). Lysozyme expressed by chicken macrophages has attracted some attention, since it is progressively activated during macrophage differentiation and has therefore been used as a stage-specific marker. Enzyme expression has been found to be low in myeloblasts but high in mature macrophages (Grewal *et al*., 1992), where it is expressed constitutively (Hauser *et al*., 1981). Furthermore, LPS stimulation of HD11 cells leads to lysozyme gene activation, which may play a role in the antibacterial response of macrophages (Goethe and Loc, 1994; Phi-van, 1996).

Respiratory Burst Activity

Release of reactive oxygen and nitrogen intermediates has been recognized as an important microbicidal mechanism of activated macrophages. Neutrophils and macrophages reduce oxygen to superoxide, a reaction catalyzed by the NADPH oxidase. O_2^- further reacts with itself to form H_2O_2 from which highly reactive oxidants such as HOCl can be formed (Babior, 1995, 2004). Stimulation of elicited chicken macrophages with phorbol ester has been shown to induce the release of high levels of superoxide (Golemboski *et al*., 1990; Lin *et al*., 1992), an effect also found with HD11 (Golemboski *et al*., 1990; Xie *et al*., 2002), MQ-NCSU (Withanage *et al*., 2005) and HTC cells (Rath *et al*., 2003). Respiratory burst activity can also be induced with phorbol myristic acid, zymosan A, a calcium ionophore (Desmidt *et al*., 1996) and different

Salmonella serotypes (Chadfield and Olsen, 2001). Respiratory burst activity to *Salmonella* serovar Gallinarum has been shown to be genetically determined and to correspond to a higher rate of elimination of intracellular bacteria (Wigley *et al*., 2002).

NO Production

In 1987, NO was identified as a highly potent anti-microbial effector molecule secreted by activated macrophages (Hibbs *et al*., 1987). Subsequent work showed that NO is generated from L-arginine, which is converted to L-citrulline and NO by the action of nitric oxide synthase (NOS). NOS exists in three isoforms, the constitutively expressed neuronal NOS (nNOS), the endothelial isoform (eNOS) and the inducible enzyme (iNOS), which is now referred to as NOS II. The constitutive isoform produced in low amounts is a physiological signalling molecule involved in neurotransmission and regulation of the vascular tone. In contrast, macrophages produce high amounts of NO in response to inflammatory stimuli and cytokines by upregulation of NOS II activity. Studies using NOS II deficient mouse models have clearly demonstrated the anti-microbial potential of this highly reactive radical (MacMicking *et al*., 1997). NO released by macrophages is rapidly converted to NO_2^- and NO_3^- at a ratio of 3:2, making direct measurement of NO technically demanding. In contrast, NO-derived NO_2^- can easily be quantified using the so-called Griess reaction (Ding *et al*., 1988), a method widely applied in avian macrophage research.

Dietert and colleagues were the first to demonstrate NO production by chicken macrophages (Sung *et al*., 1991). Stimulation of Sephadex-elicited macrophages or HD11 cells by LPS led to the secretion of maximal NO_2^- levels into the cell culture medium within 24 h. This response was shown to be L-arginine dependent, since its depletion completely prevented NO_2^- accumulation, and NOS II activity could be inhibited by L-arginine analogues (Sung *et al*., 1991). Since the chicken is unable to synthesize L-arginine from ornithine due to a lack of carbamoyl phosphate synthase I and ornithine transcarbamoylase activity in the urea cycle (Tamir and Ratner, 1963; Boorman and Lewis, 1971), this metabolic pathway depends on the nutritional supply of L-arginine. This assumption has been confirmed by the demonstration of impaired NO formation by macrophages from birds kept on an L-arginine-restricted diet (Kwak *et al*., 2001). As in mammals, chicken NOS II requires tetrahydrobiopterin as a cofactor (Sung *et al*., 1994) which is synthesized from GTP in a well-defined enzymatic pathway (Nichol *et al*., 1985). NOS II activity and tetrahydrobiopterin synthesis are induced in a coordinated way in chicken macrophages in response to stimulation with LPS or IFN-γ (Kaspers *et al*., 1997). Molecular cloning of chicken NOS II revealed between 66% and 70% protein sequence identity with mouse and human NOS II and led to the identification of several conserved transcription factor binding sides. The importance of the nuclear factor-kappa B (NFκB) pathway for NOS II induction by LPS has been clearly demonstrated using NFκB inhibitors (Lin *et al*., 1996). This work has subsequently been confirmed by others and extended to demonstrate a common signalling pathway for LPS and CpG oligodeoxynucleotide (ODN)-mediated induction of NOS II involving activated protein kinase C and mitogen-activated protein kinases. A distinctive feature of CpG ODN-mediated NO production is the requirement for clathrin-dependent endocytosis and endosomal maturation, consistent with observations on mammalian macrophages (He and Kogut, 2003).

Measurement of NOS II activity in chicken macrophages has become a frequently used method for the analysis of avian macrophage activation due to the simplicity of NO_2^- quantification. Based on this assay chicken IFN-γ was identified by functional expression cloning and shown to be the major macrophage-activating factor secreted by mitogen-stimulated lymphocytes (Digby and Lowenthal, 1995; Weining *et al*., 1996), while chicken IFN-α was finally identified as a true type I IFN with little, or no, macrophage-activating effects on its own (Schultz *et al*., 1995). However, chicken IFN-γ and chicken IFN-α were shown to potentiate NO secretion by HD11 cells synergistically (Sekellick *et al*., 1998). Similarly, turkey bone-marrow-derived macrophages do not respond to type I IFN or LPS alone but produce NO if activated by a combination of these factors (Suresh *et al*., 1995), while turkey IFN-γ was

shown to be a potent macrophage-activating factor on its own (Lawson *et al*., 2001). It should be noted that the latter experiment was performed with a transformed turkey cell line, which may not respond to cytokine treatment in the same way as primary macrophages. Nevertheless, these studies have identified IFN-γ as a potent activator of avian macrophages suggesting an important role for this cytokine in macrophage-mediated protection from pathogenic micro-organisms.

NO secretion has also been used as a readout system for the identification of pathogen-associated molecular patterns (PAMP) with macrophage-activating activity. From the initial studies on NO biology in chickens, it became clear that LPS is a potent inducer of NO production (Sung *et al*., 1991) and NOS II transcription (Lin *et al*., 1996). Interestingly, repeated stimulation of chicken macrophages with LPS *in vitro* or *in vivo* induces a complete block of NO production (Chang *et al*., 1996). This macrophage response has already been demonstrated in mammals and is known as endotoxin tolerance.

Comparison of LPS derived from different Gram-negative bacteria revealed only moderate (Dil and Qureshi, 2002) or no (Okamura *et al*., 2005) differences in their NO-inducing activities. On the other hand, macrophages obtained from chickens with different genetic backgrounds exhibit significantly different NOS II expression activities in response to LPS, thus defining hyper- and hypo-responder lines (Hussain and Qureshi, 1997, 1998). This observation has been attributed in part to differences in cell surface expression levels of the LPS receptor, Toll-like receptor (TLR)4, using anti-human TLR4 antibodies (Dil and Qureshi, 2002). However, this interpretation needs further verification with the chicken TLR4-specific tools currently under development.

Bacterial DNA contains sequence motifs termed CpG ODN which have immunostimulatory properties in mammals (Mutwiri *et al*., 2003). CpG ODN have also been shown to induce NOS II activity in chicken macrophage cell lines (Xie *et al*., 2003) and primary chicken macrophages (He *et al*., 2003, 2006). Both the optimal stimulatory sequence motif ODN 2006 for human cells and the optimal mouse ODN 1826 activate chicken HD11 cells, but ODN 1826 has been reported to be less potent (Xie *et al*., 2003). Comparison of several CpG ODN identified the GTCGTT motif as the most active NO inducer (He *et al*., 2003). It is interesting to note that while CpG ODN-mediated activation of macrophages and other chicken immune cells (Kogut *et al*., 2006) has been shown independently by several studies the CpG ODN receptor, TLR9, has not so far been identified in the chicken genome (Yilmaz *et al*., 2005). Functional studies employing reporter gene assays and expression of recombinant chicken TLR should help to identify the chicken homologue of TLR9, an approach recently taken to investigate ligand specificity of chicken TLR3 and TLR7 (Schwarz *et al*., 2007).

Besides LPS and CpG ODN lipoteichoic acid (LTA), a TLR2 agonist, was shown to induce moderate amounts of NO in monocyte-derived macrophages from three 3-day-old chicks. In contrast, the tested agonists of TLR2, TLR3, TLR5, TLR7, Pam3CSK4, polyinositol cytosine, *S. enterica* Typhimurium flagellin and loxoribine showed very weak, or no, stimulating properties (He *et al*., 2006). This finding contrasts with the observation that, with the exception of TLR5, all TLR are expressed in these cells. In addition to cytokines and TLR agonists, CD40 ligand-mediated activation of macrophages also leads to NO production (Tregaskes *et al*., 2005).

Cytokine Response of Avian Macrophages

Macrophages residing in the tissues are the first cells to encounter pathogens (see Chapter 7). Detection of micro-organisms through pattern recognition receptors not only triggers the uptake and destruction of the invaders, but also induces the secretion of signalling molecules which help to activate additional arms of the defence system. These molecules include a range of cytokines and chemokines which act locally or on a systemic level (details of chicken cytokines and chemokines can be found in Chapter 10).

Early studies addressing cytokine secretion by avian macrophages have relied on biological assays for cytokine detection and have indicated the release of IL-1 (Klasing and Peng, 1987), IL-6 (Amrani *et al*., 1986) and tumour necrosis factor (TNF)-α like factor (Klasing, 1991;

Rautenschlein *et al*., 1999) in response to inflammatory stimuli. However, a more detailed characterization of the macrophage cytokine response was not achieved until the first avian inflammatory cytokines had been cloned. It is now well established that activation of chicken macrophage cell lines and primary macrophages with different PAMP induce the release of IL-1 (Weining *et al*., 1998; Gyorfy *et al*., 2003) and IL-6 (Schneider *et al*., 2001; Rath *et al*., 2003; Smith *et al*., 2005). Interestingly, attempts to identify an avian homologue of TNF-α in the chicken genome have failed (Kaiser *et al*., 2005) despite the repeated demonstration of a TNF-like activity secreted by macrophages (Zhang *et al*., 1995; Rautenschlein *et al*., 1999). This discrepancy may be partially explained by the identification of chicken TL1a, a TNF family member related to TNF-α, i.e. upregulated in the spleen in response to LPS treatment. Recombinant TL1a exhibits cytotoxic activity against L292 target cells and chicken fibroblasts (Takimoto *et al*., 2005) and is induced in HD11 cells by treatment with the recently identified protein LPS-induced TNF-α factor (LITAF; Hong *et al*., 2006).

In addition to pro-inflammatory cytokines, LPS-activated HD11 cells and monocyte-derived macrophages both express the anti-inflammatory cytokine IL-10 and may thereby exert multiple immunoregulatory effects during infections (Rothwell *et al*., 2004).

RT-PCR and quantitative RT-PCR techniques are now increasingly used to investigate the cytokine and chemokine responses of avian macrophages to whole micro-organisms and defined microbial products. HD11 cells infected with *C. jejuni* were shown to respond with significantly increased mRNA levels for IL-1 and IL-6 and for the two IL-8-like chemokines 9E3/CEF4 and K60 (CXCLi1; Smith *et al*., 2005). Similarly, increased expression of pro-inflammatory cytokines (IL-1 and IL-6) and chemokines (CCLi2 and CXCLi1) was observed in macrophages infected with either *S. enterica* serovar Gallinarum or Typhimurium. In this study, significant differences in the cytokine expression level and pattern were observed between macrophages derived from resistant and susceptible chicken lines. This included differences in IL-18 mRNA abundance which may in part explain the resistant phenotype, since IL-18 is involved in the induction of IFN-γ, a critical cytokine in the control of *Salmonella* infections (Wigley *et al*., 2006).

The induction of IL-1, IL-6, IL-18 and chemokine genes seems to be a uniform response pattern of chicken macrophages to microbial stimuli, since it was not only observed in response to bacteria, but also after viral (Palmquist *et al*., 2006) and parasitic infection (Dalloul *et al*., 2007) of macrophage cultures. Application of microarray analysis is a promising approach to identify new genes that are differentially regulated in macrophages in response to different pathogens. For this purpose, a macrophage-specific complementary (c)DNA microarray has been constructed and used to compare the response of monocyte-derived macrophages to *E. coli* and LPS. Significant changes in the expression levels have been observed for 981 and 243 genes in response to *E. coli* and LPS, respectively, clearly demonstrating the magnitude of macrophage reactions and the problems associated with data analysis and interpretation (Bliss *et al*., 2005). This array has also been used to study the macrophage response to *Eimeria* parasites in more detail, and has led to the identification of an extended list of chicken cytokines, differentially regulated after infection with different *Eimeria* species (Dalloul *et al*., 2007).

FUNCTIONAL PROPERTIES OF CHICKEN DC

Maturation from Antigen Sampling to Antigen Presenting

Both avian *ex vivo* and bone-marrow-derived DC are capable of phagocytosis and macropinocytosis (C. Butter and Z. Wu, unpublished observations) which is much reduced on stimulation with CD40 ligand or LPS. Immature DC also express high levels of the endocytosis receptor, DEC205 (K. Staines, personal communication). These properties contribute to efficient antigen sampling, observed with mammals where, to effectively present antigen, DC require concentrations of antigen between 10^3 and 10^6 less than other APC.

In common with mammalian systems, avian bone-marrow-derived DC matured *in vitro* lose their ability to take up antigen efficiently and upregulate molecules important for antigen

presentation, including MHC antigens and CD40 (Z. Wu, personal communication). Matured DC, either bone marrow or *ex vivo* spleen or lung derived, will stimulate allogeneic T cells in a mixed lymphocyte reaction.

Migration

DC from skin and lung can migrate to the spleen following the uptake of fluorescent antigen (C. Butter, unpublished observations). The epidermal DC described by Igyarto *et al.* (2006) can be mobilized from the epidermis by hapten treatment of the epidermis *in vivo*. This results in a decrease in the number of the Langerhans cells in the dermis with the concurrent appearance of hapten-positive cells in the so-called dermal lymphoid nodules. This suggests that these dermal nodules maintain some regional immunological function, similar to the mammalian lymph nodes.

The location of antigen presentation in avian species which lack mammalian-like lymph nodes remains to be fully elucidated. Although the spleen has a role in mounting responses to antigens administered intravenously, responses to antigens given locally appear to be initiated elsewhere (Hippelainen and Naukkarinen, 1990). Early work described the presence of lymphoid nodules in the chicken (Biggs, 1957) and subsequent studies have identified a number of novel lymphoid structures and aggregates including in the lung (Olah and Glick, 1984; Jeurissen *et al.*, 1989; Olah *et al.*, 2003, Reese *et al.*, 2006; Chapter 2). Their role in mounting immune responses remains to be determined.

CONCLUDING REMARKS

Avian macrophage biology has been studied in much detail *in vitro* utilizing transformed cell lines or primary macrophage cultures. When interpreting and comparing results, one should keep the obvious limitations of cell culture techniques in mind. Cell lines are convenient tools for cell biology studies, however, they represent only a single stage of differentiation within the myelomonocytic lineage, and cell lines representing a range of differentiation states are not available. Furthermore, transformation has a significant impact on the functional properties of such cells and results obtained from studies with cell lines may not properly reflect macrophage function. On the other hand, primary macrophage cultures are more likely to be heterogeneous and isolation of these cells should lead to their activation and differentiation. Finally, the efficacy of monocyte and macrophage isolation and the cellular responses may be different when using different chicken lines, as demonstrated in the *Salmonella* model (Wigley *et al.*, 2006).

As the repertoire of readout systems for macrophage and DC function increases with progress in gene identification and gene expression technology, our understanding of the role of these cells in the avian immune system will improve. Importantly, in future, new tools and techniques need to be developed that will enable specific identification of macrophage and DC subsets *in situ* and for functional studies to be done *in vivo*; by depletion, transgene technologies or gene silencing approaches. First steps into this process have been made with the publication of a macrophage depletion method (Rivas *et al.*, 2003). Recent advances in the production of tools for the identification and generation of avian DC presage a rapid expansion of knowledge in this crucial area of avian immunobiology.

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10 AVIAN CYTOKINES AND CHEMOKINES

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DEFINITIONS
DESCRIPTION OF DIFFERENT AVIAN CYTOKINE AND CHEMOKINE FAMILIES
CYTOKINE CASCADES
CYTOKINE RECEPTORS
REAGENTS AVAILABLE
ASSAY SYSTEMS AVAILABLE
REGULATION OF CYTOKINE RESPONSES
VIRAL PROTEINS BLOCKING CYTOKINE ACTION
POTENTIAL USE OF CYTOKINES AS VACCINE ADJUVANTS
IMPROVED VACCINES BASED ON VIRAL MUTANTS LACKING CYTOKINE ANTAGONISTS
REFERENCES

DEFINITIONS

The term "cytokine" was first coined by Cohen *et al*. (1974). Cytokines are regulatory peptides, with molecular weights typically less than 30 kDa, that act as extracellular signals between cells in immunological development and during the course of immune responses. They both elicit and regulate immune responses, and can be produced by virtually every cell type, having pleiotropic effects on cells of the immune system, as well as modulating inflammatory responses. Historically, they have been variously described as lymphocyte-derived factors known as "lymphokines", monocyte-derived factors called "monokines", haematopoietic "colony-stimulating factors" and connective tissue "growth factors". However, for the purpose of this chapter we will use a tighter definition of "cytokine", to refer to regulatory molecules, generally secreted but sometimes acting as cell surface molecules, that are either produced by, or exert their effects on, cells of the immune system. Under this definition, we will specifically discuss the interleukins (IL), the interferons (IFN), the transforming growth factor-β (TGF-β) family, the tumour necrosis factor (TNF) superfamily (TNFSF), the colony-stimulating factors (CSF) and the chemokines.

Simply put, the IL series have functional activities involving lymphocytes and the IFN series have antiviral effects. The roles of TGF-β, TNFSF and CSF are obvious, but it must be remembered that the TGF-β family has a crucial role in regulating inflammatory reactions, and the TNFSF have a wide range of activities unrelated to any antitumour activity. A further, and important, group of regulatory molecules is the chemokines, whose primary function is to regulate leukocyte traffic.

DESCRIPTION OF DIFFERENT AVIAN CYTOKINE AND CHEMOKINE FAMILIES

Our understanding of the repertoire of cytokines and chemokines in the chicken was limited until recently, compared to those of mammalian species. As recently as a decade ago, only the

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type I IFN (Sekellick *et al*., 1994; Sick *et al*., 1996) and the TGF-β family (Jakowlew *et al*., 1988, 1990; Burt and Jakowlew, 1992) had been characterized in the chicken. In general, chicken cytokines have only 25–35% amino acid identity with their mammalian orthologues. As a result, there are few, if any, cross-reactive monoclonal antibodies (mAbs) or bioassays. Moreover, cross-hybridization or degenerative RT-PCR/PCR approaches also have been unsuccessful. Prior to the release of the chicken genome sequence (International Chicken Genome Sequencing Consortium, 2004), some progress had been made through a combination of expression cloning from expressed sequence tag (EST) libraries (Digby and Lowenthal, 1995; Sundick and Gill-Dixon, 1997; Weining *et al*., 1998), systematic sequencing of EST libraries (Schneider *et al*., 2000, 2001; Lillehoj *et al*., 2001; Min and Lillehoj, 2002, 2004; Rothwell *et al*., 2004) and genomics approaches based on the conservation of synteny (Balu and Kaiser, 2003; Avery *et al*., 2004). However, the availability of the chicken genome sequence has radically altered our ability to understand both the repertoire (Kaiser *et al*., 2005) and thereafter the biology of the avian cytokines and chemokines. As will be discussed below, many of the cytokines and chemokines identified in mammals are also present in the chicken. The exceptions are in multigene families of cytokines and chemokines, where the chicken seems to have fewer members than in the equivalent families in mammals. The absence of some of these multigene family members may explain some of the fundamental differences in the organs and cells of the avian immune system, as shall be discussed below.

New members of cytokine gene families in mammals have continued to be discovered even after the advent of complete genome sequences for man and mouse. These include new members of the IL-1, IL-10 and IL-17 families. In some cases, as more knowledge becomes available about the biological functions and structural properties of these new cytokines, there have been nomenclature changes to reflect this. Hence, IL-28A, IL-28B and IL-29 have recently been shown to constitute a new family of type I IFN (see below) and have been renamed IFN-λ1–3 (Kempuraj *et al*., 2004).

The Interleukins

IL-1 Family

In man, the IL-1 family originally included IL-1α, IL-1β, IL-1RN (receptor antagonist) and IL-18. Since the human genome sequence became available, the family has expanded and includes six new genes encoding novel IL-1 homologues (IL-1F5 to IL-1F10), which lie next to the other three IL-1 genes on chromosome 2 (Fig. 10.1(a)). IL-18, however, lies on chromosome 11. The functions of the novel IL-1 family members are not fully understood, but at least some have a role in inflammatory reactions. Both IL-1β (Weining *et al*., 1998) and IL-18 (Schneider *et al*., 2000) have been cloned in the chicken, and both have roles in inflammatory reactions. In the new genome sequence assembly (v2.0; ftp://genome.wustl.edu/pub/user/lhillier/private/chicken_060412.tar.gz), the gene for IL-1β is present on chromosome 4, although previously it had been mapped to chromosome 2 (Kaiser *et al*., 2004). None of the other IL-1 family members are identifiable in the chicken genome (Kaiser *et al*., 2005). Whilst there may be other IL-1 family members in the chicken (in particular, it is surprising that IL-1α and IL-1RN are apparently absent), it is likely that the chicken has fewer IL-1 family members than mammals.

T Cell Proliferative Interleukins

The genes encoding the T cell proliferative interleukins IL-2 (Sundick and Gill-Dixon, 1997), IL-15 (Lillehoj *et al*., 2001) and IL-21 (Kaiser *et al*., 2005) all lie on chicken chromosome 4, although they are not syntenic. The biological activities of both IL-2 and IL-15 have been well characterized (Stepaniak *et al*., 1999; Kolodsick *et al*., 2001; Lillehoj *et al*., 2001; Hilton *et al*., 2002a; Kogut *et al*., 2002). Chicken IL-21 is expressed (T. Poh, L. Rothwell and P. Kaiser, unpublished observations), but nothing has yet been published on its biological activities.

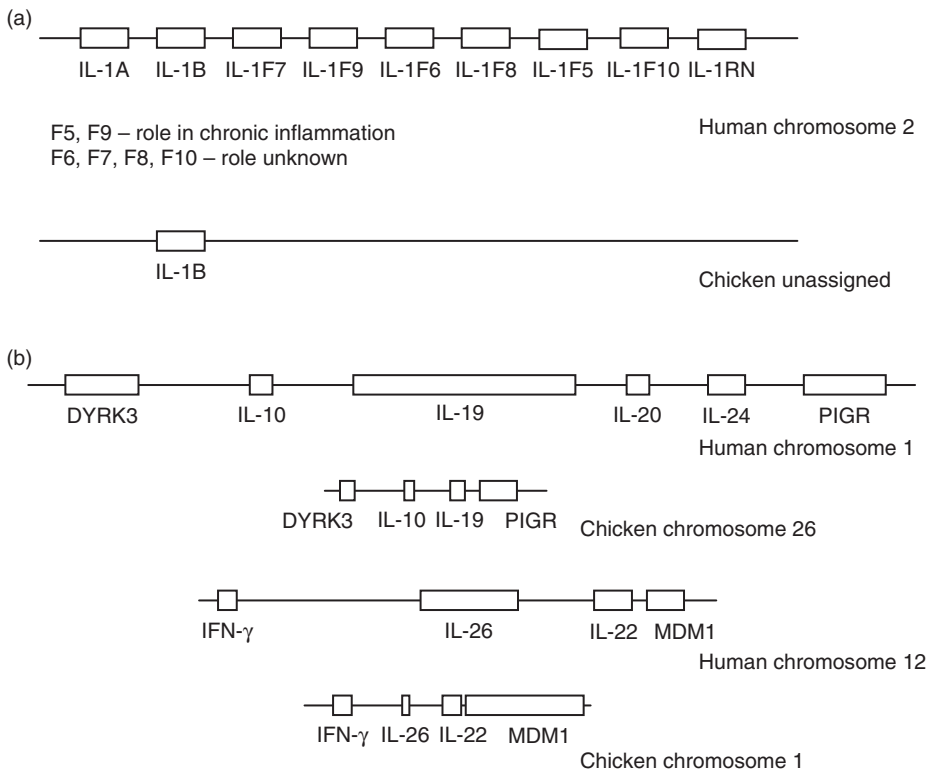

FIGURE 10.1 Schematic drawings comparing the human and chicken IL-1 and IL-10 gene families. (a) IL-1: there are nine IL-1β family member genes (IL-1A, IL-1B, IL-1RN and IL-1F5–F10) syntenic on human chromosome 2, but only a single unassigned IL-1B gene identified to date in the chicken genome (genes not drawn to scale). For most of the new IL-1 family members in man, function is unknown, but IL-1F5 and IL-1F9 have a role in inflammation. (b) IL-10: there are six IL-10 family member genes in human. Four (IL-10, IL-19, IL-20 and IL-24) syntenic on chromosome 1, the other two (IL-22 and IL-26) syntenic with IFN-γ on chromosome 12. There are also two clusters of IL-10 family genes in the chicken: IL-22 and IL-26 syntenic with IFN-γ on chromosome 1, and IL-10 and IL-19 on chromosome 26.

Th1 Interleukins

The biological role of IL-12 in driving inflammatory Th1 responses has long been characterized. Chicken genes encoding the two components of IL-12, IL-12α (p35; Degen *et al*., 2004) and IL-12β (p40; Balu and Kaiser, 2003; Degen *et al*., 2004) were recently cloned and the biological activity of IL-12 shown to be similar to that in mammals.

In man and mouse, it has recently become apparent that there are at least two other members of the IL-12 family, IL-23 and IL-27, that also regulate responses of Th cells. IL-12 and IL-23 are both heterodimers, share a common subunit, p40, and bind to a common receptor subunit, IL-12Rβ1. However, each cytokine has distinct functions *in vivo*, and their receptors also have a ligand-specific subunit. IL-23, in particular, drives differentiation of Th17 cells (see below). IL-27 is also a heterodimer, in this case made up of EBI3, an IL-12 p40-related protein, and p28, an IL-12 p35-related protein. It has both pro-inflammatory and anti-inflammatory properties. Although there is no evidence of specific genes for IL-23 or IL-27 in the chicken genome, there is an IL-23R gene on chromosome 8 (Kaiser *et al*., 2005). Considering the role of IL-23 in driving IL-17A and IL-17F production in mammals, and the existence of these two IL-17 family member genes in the chicken (Kaiser *et al*., 2005), it seems highly likely that there is a chicken orthologue of IL-23 yet to be identified.

Th2 Interleukins

The Th2 cytokine gene cluster is an exception to the rule for multigene families in the chicken. For all of the other multigene families, the chicken has fewer members than the equivalent families in mammals. For the Th2 cytokines, the chicken possesses an extra family member not yet identified in mammals. So, as well as the canonical Th2 cytokine gene cluster genes IL-3, IL-4, IL-5, IL-13 and granulocyte macrophage colony-stimulating factor (GM-CSF) (Avery *et al*., 2004), the chicken cluster also encodes another cytokine-like transcript, KK34 (Koskela *et al*., 2004a), which is differentially expressed in chicken γδ T cells. As yet, no orthologue of KK34 has been described in mammals.

The Th1–Th2 Paradigm

The Th1–Th2 paradigm in biomedical species has been central to our understanding of the adaptive immune response. Mammals have an immune system able to polarize functionally into type 1 or type 2 immune pathways that resolve infections with intracellular or extracellular pathogens, respectively (Janeway, 1992). The polarization of the responses is largely regulated by antigen-specific Th cells. Th1 cells drive cell-mediated, inflammatory responses, and Th2 cells drive responses against helminthic worms and allergy (humoral immunity). Th1 cells typically produce IFN-γ, driven by early production of IL-12 and IL-18. Th2 cells typically produce IL-4, IL-5, IL-9, IL-13 and IL-19, driven by IL-4 or IL-13. Recently, a third lineage of Th cells, Th17, has been identified, that produce IL-17 under the influence of IL-23, which is closely related to IL-12 (see above).

Until recently, the existence of the Th1–Th2 paradigm outwith mammalian species was not known. When compared with mammals, certain components of the humoral immune response in chickens are absent. For example, chickens lack IgE and subclasses of IgY (the avian homologue of IgG); functional eosinophils appear to be absent; the eotaxins and the eotaxin receptor are absent (see below); IL-5 is apparently a pseudogene (Kaiser *et al*., 2005) and Th2-associated allergies have not been described for birds. However, the paradigm was initially couched in terms of the cytokine profiles that dominate after infection with intracellular or extracellular pathogens. The cytokine response to an intracellular pathogen is predominated by IFN-γ, and to an extracellular pathogen by IL-4 and IL-13. We recently showed (W.D.J. Degen *et al*., 2005) that the response to Newcastle disease virus (NDV) was dominated by IFN-γ and that to an ascarid worm, *Ascaridia galli*, was dominated by IL-4 and in particular IL-13. We have since extended these studies to infections with infectious bursal disease virus (IBDV; Eldaghayes *et al*., 2006; L. Rothwell and P. Kaiser, unpublished observations) and *Histomonas meleagridis* (F. Powell, M. Clarkson and P. Kaiser, unpublished observations), and shown that the responses to these infections are dominated by IFN-γ and IL-4, IL-13 and IL-19, respectively.

In our opinion, this is compelling evidence of the polarization of type 1 and type 2 adaptive immune responses extending beyond mammalian species to galliforms, at least. It remains to be determined if this paradigm holds at the cellular and molecular levels, and if avian Th cells can become terminally polarized to a Th1 or Th2 phenotype.

IL-10 Family

In man, the IL-10 family comprises six members, encoded in two clusters on different chromosomes. IL-10, IL-19, IL-20 and IL-24 are syntenic on chromosome 1, while IL-22 and IL-26 are syntenic on chromosome 12. By comparison, the mouse has five members, lacking IL-26. The chicken has only four members of this family: IL-10 and IL-19 on chromosome 26, and IL-22 and IL-26 on chromosome 1 (Fig. 10.1(b)). All four chicken genes are expressed (Rothwell *et al*., 2004; U. Pathania and P. Kaiser, unpublished observations). The function of IL-10 appears to be conserved in the chicken, in that it acts as an anti-inflammatory cytokine, downregulating the effects of IFN-γ (Rothwell *et al*., 2004). In mammals, IL-19 is a Th2 cytokine, whereas IL-22 and IL-26 are involved in inflammatory responses. For IL-19 and IL-22 at least, these functions seem to be conserved in the chicken. IL-19 is expressed along with IL-4 and IL-13

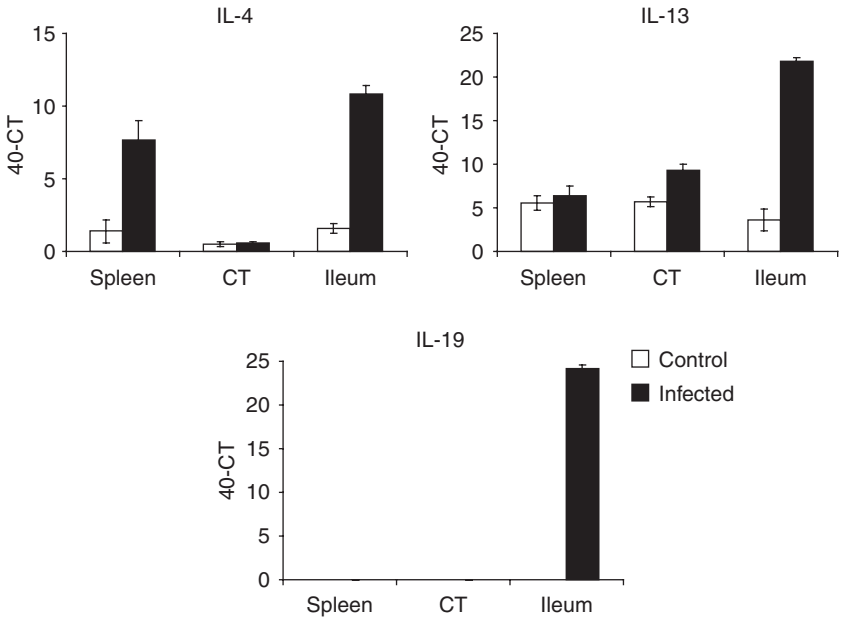

FIGURE 10.2 Expression of mRNA for Th2 cytokines (IL-4, IL-13 and IL-19) in the spleen, caecal tonsil (CT) and ileum following oral infection with 1000 embryonated *A. galli* (strain Liederbach) worm eggs or in age-matched uninfected controls, as measured by real-time quantitative RT-PCR, with results expressed as 40-Ct values ± SE. The infection experiments were carried out by Winfried Degen, Nancy Daal and Virgil Schijns of the Department of Vaccine Technology and Immunology, Intervet International BV, The Netherlands.

following infections with extracellular pathogens such as worms (*A. galli*; Fig. 10.2) or protozoa (*H. meleagridis*; F. Powell, M. Clarkson and P. Kaiser, unpublished observations). In mammals, IL-22 upregulates the expression of β-defensins in keratinocytes (Wolk *et al.*, 2004) and induces IL-10 expression in epithelial cells (Nagalakshmi *et al.*, 2004). Chicken IL-22 induces IL-10 expression in chicken kidney cells, the best available chicken epithelial cell model (Fig. 10.3) and upregulates the expression of β-defensins in heterophils (U. Pathania and P. Kaiser, unpublished observations).

IL-17 Family

Unlike the other cytokine multigene families discussed in this chapter, the IL-17 family in mammals is not syntenic but, in general, is dispersed in the genome. Of the six IL-17 family members described for man (IL-17A–F), four are readily identified in the chicken genome: IL-17A, IL-17B, IL-17D and IL-17F (Kaiser *et al.*, 2005; Table 10.1). IL-17, whose biological functions have been characterized in the chicken (Min and Lillehoj, 2002), is synonymous with IL-17A. In man and mouse, there is a lineage of Th cells, Th17, that selectively produce IL-17A (see above), and are thought to be key regulators of inflammation (Chen *et al.*, 2006; Harrington *et al.*, 2006). Expression of IL-17A and IL-17F, at least, are under the control of IL-23, a cytokine related to IL-12 (see above). It will be interesting to understand the function of the IL-17 family members in the chicken, in particular to understand the lineages of Th cells that the chicken possesses.

Other Interleukins

There are, of course, other IL which do not lie in multigene families, but nevertheless play a vital role in the chicken's immune response. One of the first IL characterized in the chicken was IL-6 (Schneider *et al.*, 2001). It is a pro-inflammatory cytokine in both chickens and mammals and is produced early after infection as part of the induced innate immune response.

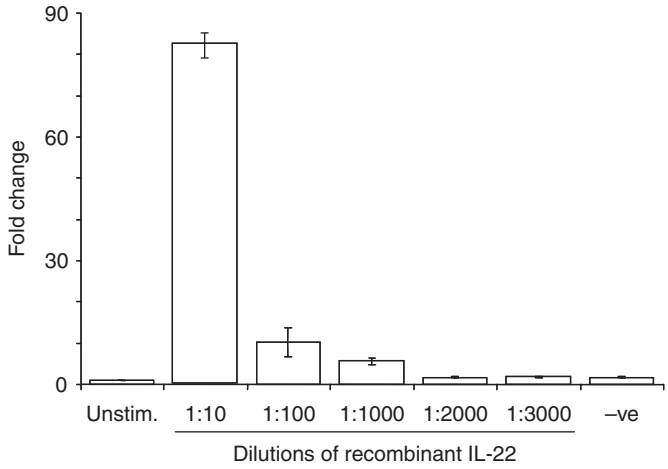

FIGURE 10.3 Recombinant chicken IL-22 stimulates IL-10 mRNA expression in chick kidney cells (CKC) in a dose-dependent manner. Fold changes in mRNA levels shown as compared to levels in unstimulated CKC (unstim.). Recombinant IL-22 = dilutions of supernatant from COS cells transfected with pCIneo expressing IL-22. -ve = CKC stimulated with supernatant from COS cells transfected with the expression vector (pCIneo) alone.

TABLE 10.1 The IL-17 Family Members in Human and Chicken, With Their Chromosomal Locations

Human	Chromosome	Chicken	Chromosome
IL-17A	6	IL-17A	3
IL-17B	5	IL-17B	13
IL-17C	16		
IL-17D	13	IL-17D	1
IL-17E	14		
IL-17F	6	IL-17F	3

IL-7 (Kaiser *et al*., 2005), IL-9 (Kaiser *et al*., 2005) and IL-16 (Min and Lillehoj, 2004) are all encoded in the chicken genome and are all expressed. IL-16 in both chickens and mammals is an inflammatory cytokine that has chemotactic activity for splenic lymphocytes (Min and Lillehoj, 2004). Although no gene for IL-11 is immediately evident in the chicken genome sequence, there is an IL-11R α chain gene on the Z chromosome (Kaiser *et al*., 2005), implying that this cytokine will also be present.

The Interferons

Type I IFN

There are three subgroups of type I IFN so far identified in the chicken: IFN-α, IFN-β and IFN-λ. In the chicken, IFN-α and IFN-β both have antiviral activity (Sekellick *et al*., 1994; Sick *et al*., 1996). As in mammals, IFN-α is a family of intronless genes, whereas IFN-β is a single-copy intronless gene present on the same chromosome (Z chromosome in the chicken). Assembly of the sex chromosomes in the chicken genome sequence is poor, and currently it is not clear how many IFN-α genes are actually present. To date, full or partial genes for at least seven IFN-α species can be identified. Three of these have been cloned and fully sequenced as cDNA.

In man, the three IFN-λ genes are all clustered together on chromosome 19. The syntenic region in the chicken is on chromosome 7, where only a single IFN-λ gene can be identified (Kaiser *et al*., 2005). This gene is expressed (T. Hu, L. Rothwell and P. Kaiser, unpublished observations), but its biological activity is yet to be determined.

Type II IFN

The chicken IFN-γ gene was identified some years ago (Digby and Lowenthal, 1995) and its biological activities, role in the immune response and potential as a vaccine adjuvant (see later) have been well documented in the literature. As described above, it is the key signature cytokine of Th1-controlled responses, and crucial in controlling infections with intracellular pathogens.

TGF-β Family

The TGF-β family, in chickens and mammals, plays an important role in immunoregulation. Despite there being four chicken TGF-β genes identified in the literature and databases, most avian immunologists now accept that, in reality, there are really only three TGF-β genes in the chicken, as in mammals. There are direct orthologues of mammalian TGF-β2 and TGF-β3 (Jakowlew *et al*., 1988, 1990), whereas the chicken orthologue of mammalian TGF-β1 is chicken TGF-β4 (Burt and Jakowlew, 1992; Jakowlew *et al*., 1997; Pan and Halper, 2003). In mammals, TGF-β1 is primarily an anti-inflammatory cytokine, although under certain conditions it can be pro-inflammatory. TGF-β4 also has anti-inflammatory properties (Kogut *et al*., 2003; Secombes and Kaiser, 2003; Swaggerty *et al*., 2004).

Tumour Necrosis Factor Superfamily

Members of the TNFSF and TNF receptor (TNFR) superfamily (TNFRSF) have crucial roles in both innate and adaptive immunity, including in inflammation, apoptosis, cell proliferation and stimulation of the immune system. TNFSF proteins are homotrimeric in structure. Most of the TNFSF members should really be considered as co-stimulatory molecules, rather than cytokines, and are discussed in more detail in Chapter 5. The TNFSF members which can be considered as cytokines are TNF-α, lymphotoxin (LT)-α, LT-β and B cell activating factor of the TNF family (BAFF). Chicken BAFF has been cloned independently in two laboratories (Koskela *et al*., 2004b; Schneider *et al*., 2004) and, as in mammals, mediates B cell survival.

Our analysis of the chicken genome (Kaiser *et al*., 2005) identified certain TNFSF members, but others were absent (Table 10.2). Bearing in mind that the chicken genome is not complete, and therefore a significant proportion of genes may not be represented, we also analysed the genome for the TNFRSF members (Table 10.2). If both a TNFSF member and its cognate receptor were absent, this could be taken as additional evidence for the non-existence of that specific TNFSF member in the chicken. Receptors were found for all of the TNFSF members so far identified. Of the eight missing members, receptors for only two were found.

In mammals, TNF-α, LT-α and LT-β are clustered together in the class III region of the major histocompatibility complex (MHC). There is no evidence for any of these three TNFSF members in the chicken MHC, or indeed in the chicken genome sequence. In fish genomes, there is no evidence for LT genes, but there are apparent TNF-α genes. We have identified a potential TNFRSF2 gene, the receptor for TNF-α, in the chicken genome, suggesting TNF-α may be present although as yet unidentified. There have also been several reports of TNF-α-like activity in chickens.

In mammals, it has been shown that the LT genes are crucial for the development of secondary lymphoid organs, including lymph nodes (Fu and Chaplin, 1999; Ruddle, 1999). It seems a reasonable hypothesis that the lack of lymph nodes in the chicken might be due to the lack of LT genes.

TABLE 10.2 The Tumour Necrosis Factor (TNF) Superfamily (TNFSF) and TNF Receptor (TNFR) Superfamily Members in the Chicken

TNFSF Member	Also Known as	Present in Chicken Genome	Cognate TNFR Present in Chicken Genome
1	LT-α	No	No
2	TNF-α	No	Yes
3	LT-β	No	No
4	OX40L	Yes	Yes
18	AITRL	Yes	Yes
6	FASL	Yes	Yes
9	4-1BBL	No	Yes
7	CD27L	No	No
14	LIGHT	No	No
12	TWEAK	No	No
13	APRIL	No	No
15	VEGI	Yes	Yes
8	CD30L	Yes	Yes
5	CD40L	Yes	Yes
10	TRAIL	Yes	Yes
11	RANKL	Yes	Yes
13B	BAFF	Yes	Yes
–	TRAIL-L*	Yes	?*

TNFSF genes separated by bold horizontal lines are syntenic in both human and chicken, with the exception of the last five genes, which are on separate chromosomes in each species.

*TRAIL-L = TRAIL-like. ? = TRAIL-L is a chicken-specific TNFSF member and therefore the cognate receptor is not known.

Colony-Stimulating Factors

CSF mediate the development of myeloid cells from pluripotent haematopoietic stem cells. In mammals, there are three CSF: granulocyte macrophage (GM)-CSF, granulocyte (G)-CSF and macrophage (M)-CSF.

Chicken GM-CSF lies in the Th2 gene cluster on chromosome 13 (see above), is expressed in the bone marrow and other tissues, and can stimulate the proliferation of chicken bone marrow cells (Avery *et al*., 2004). There is no evidence in the chicken genome for M-CSF – neither the gene itself nor the cognate receptor (Kaiser *et al*., 2005).

There is a gene for chicken G-CSF and a long history of data being generated on the function of this important molecule. However, none of the literature refers to chicken G-CSF, but rather to a molecule known as myelomonocytic growth factor (chicken MGF; Leutz *et al*., 1984, 1988, 1989; Sterneck *et al*., 1992). Chicken MGF has significant identity with mammalian IL-6 and G-CSF, but was long thought to be a separate molecule, that seemed to be chicken specific. The true identity of MGF only recently has become clear. Our recent cataloguing of the chicken's cytokine and chemokine repertoire (Kaiser *et al*., 2005) failed to find evidence in the genome for G-CSF. There was an unmapped region of the chicken genome with conserved synteny for genes that lie on either side of G-CSF in man and mouse, with a gap where the G-CSF gene would be expected. We also failed to find the gene for MGF in the genome. However, there was a G-CSFR gene suggesting that the cytokine is present in the chicken.

The genomes of several fish species have recently been sequenced, and fish G-CSF genes identified (Santos *et al*., 2006). Based on the high similarity between MGF and fish and mammalian G-CSF, Santos *et al*. (2006) hypothesized that MGF is actually G-CSF. They too found the contig gap which we originally reported, but searched the region with the MGF promoter (Sterneck *et al*., 1992), and found a near perfect match between the promoter (~1500 bp) and

a region just upstream of the contig gap. Based on this, they have hypothesized that MGF is actually the chicken G-CSF, although this has yet to be formally proven by closing this gap in the chicken genome sequence. Based on their analysis, and the biological properties of MGF, it does seem that it is actually the orthologue of mammalian G-CSF, and should now be renamed chicken G-CSF.

Chemokines

The immune systems of all higher vertebrates are a collection of single cells, whose immune surveillance functions depend on their ability to migrate, enter tissues and interact with one another. The chemokine system regulates this process by directing the circulation of lymphoid cells and their recruitment to sites of infection. Functionally, chemokines can be divided into two broad categories, homeostatic (constitutively expressed) and inflammatory (inducible). In the simplest terms, homeostatic chemokines are involved in the physiological trafficking of leukocytes, whilst inflammatory chemokines play a role in the recruitment of cells to sites of inflammation. Though useful, this distinction between the two classes is not absolute and there is some overlap. However, as a paradigm it does provide a means for subdividing the large number of chemokine genes.

The chemokine repertoire is thought to be derived from multiple gene duplications followed by sequence divergence. This expansion has been more prevalent in the inflammatory genes, with the generation of three major clusters in both humans and mice, whilst the homeostatic genes are found as individual genes or in small clusters.

Chemokine nomenclature splits chemokines into four groups – XC, CC, CXC and CX3C – based on the spacing of the first two conserved cysteines at the amino termini of these chemotactic proteins (the exception being XC, where the first cysteine is absent). Ligands (the chemokines themselves) are given the suffix "L", whereas their receptors are given the suffix "R".

XC and CX3C Chemokines

The chicken orthologue of mammalian XCL, or lymphotactin, has been cloned and acts as a chemoattractant preferentially for splenic B cells (Rossi *et al*., 1999). The chicken genome sequence also contains the gene for fractalkine (CX3CL), but there are currently no reports on its biological activity.

CC Chemokines

There are at least 14 CCL chemokines encoded in the chicken genome, of which 5 were not predicted by ENSEMBL. Four homeostatic CCL chemokines have been identified, with clear orthologous relationships (CCL17, CCL19, CCL20 and CCL21). The chicken apparently lacks obvious orthologues of CCL11, CCL24 and CCL26 (eotaxin 1–3), which in mammals attract eosinophils and basophils through their cognate receptor, CCR3, which is also absent in the chicken. The lack of functional eosinophils in the chicken fits with the lack of eosinophil-attracting chemokines.

The orthologous relationships of the inflammatory CCL to those in other species, including man and mouse, remain unclear. Chicken chromosome 19 encodes 10 chicken CCL (chCCL) chemokines in two clusters. One cluster contains the previously described K203 (Sick *et al*., 2000), two other macrophage inflammatory protein (MIP)-1β-like chemokines including the previously described chCCL4 (Petrenko *et al*., 1995; Hughes and Bumstead, 1999) and the previously described chCCL chemokine ah294 (Hughes *et al*., 2001), and a previously undescribed CCL gene. The other cluster contains six CCL chemokines (Kaiser *et al*., 2005). The two chicken clusters correspond to clusters of genes on human chromosome 17 and mouse chromosome 11, which encode the MIP and monocyte chemotactic protein (MCP) family of CCL chemokines, respectively. Phylogenetic analysis did not really clarify the relationship between the chicken inflammatory CCL genes and those of man and mouse, but it was clear that the chicken contains representatives, but fewer members, of both mammalian CCL groups, the MIP (four in the chicken) and MCP (six in the chicken) family chemokines.

We previously proposed that the four chicken MIP family members be named chicken chCCLi1–4, according to their position on chicken chromosome 19, until such time as further analysis could determine if any of them were direct orthologues of mammalian MIP family members (Kaiser *et al*., 2005). More recent analysis (Wang *et al*., 2005; de Vries *et al*., 2006; Hughes *et al*., 2006) suggests that chCCLi4 is the orthologue of mammalian CCL5 and that chCCLi3 (K203) may be an orthologue of human CCL16. The other two chemokines do not have obvious orthologues, and therefore we propose that they should be called chCCLi1 and chCCLi2, until their biological function is further characterized.

CXC Chemokines

For the homeostatic CXC chemokines, there appear to be five genes in the chicken. Two are apparent orthologues of CXCL12 (SDF-1) (Stebler *et al*., 2004; Read *et al*., 2005) and CXCL14 (BRAK). The other three represent an apparent expansion of an ancestral CXCL13 gene, present as only a single-copy gene in mammals. CXCL13 in mammals is a B cell chemoattractant. In mammals, the development of the B cell receptor repertoire takes place in the bone marrow. In the chicken, B cells develop initially in the spleen and bone marrow (see Chapter 3), but then migrate to the bursa of Fabricius, where the development of the B cell receptor repertoire takes place (see Chapter 4). After involution of the bursa, the bone marrow is considered to be the source of post-bursal stem cells. It therefore seems a reasonable hypothesis that the chicken has evolved an extended range of homeostatic B cell chemoattractants to direct trafficking of B cells during this more complex developmental pathway.

The chicken apparently lacks orthologues of CXCL9–11, which in mammals are induced by IFN-γ, attract Th1 and NK (natural killer) cells and are recognized by CXCR3, which is also absent from the genome.

As for the inflammatory CCL chemokines, the chicken has a different repertoire of inflammatory CXCL chemokines compared to mammals. In mammals, these CXCL are encoded in a single multigene family, with 10 members in man and 5 in the mouse. The equivalent region of the chicken genome encodes three genes. Two encode the previously described CAF and K60 (Martins-Green and Feugate, 1998; Sick *et al*., 2000). In phylogenetic analysis, these two genes group with human CXCL8, apart from the other human and mouse inflammatory CXC which group together. The third chicken inflammatory CXCL has no obvious orthologue.

Both CAF and K60 are ligands for CXCR1, the cognate receptor (T. Poh and P. Kaiser, unpublished observations). Both chemokines are expressed in lymphoid tissues. However, the expression profile for K60 is more restricted compared to that of CAF. K60 is mainly expressed in lymphoid tissues, whereas CAF is widely expressed in both lymphoid and non-lymphoid tissues, suggesting that the latter might have an immunosurveillance function (T. Poh and P. Kaiser, unpublished observations). Although both K60 and CAF are similar to human CXCL8, evidence from infection studies has also suggested that they might have different roles (Laurent *et al*., 2001; Withanage *et al*., 2004). In both published studies, K60 was upregulated to a higher level than CAF following infection. CAF induced the migration of both blood-derived mononuclear cells (at low concentrations) and heterophils (at higher concentrations) (Barker *et al*., 1993). K60 is more efficient in inducing the migration of heterophils than CAF (T. Poh and P. Kaiser, unpublished observations).

The chicken therefore appears to have evolved to have two different CXCL8-like chemokines; CAF is more efficient at inducing the migration of monocytes while K60 is more efficient at inducing the migration of heterophils. It seems a reasonable hypothesis that K60 is the orthologue of huCXCL8 and that it functions mainly as a heterophil attractant whilst CAF, although similar to huCXCL8, functions mainly to attract monocytes.

CYTOKINE CASCADES

Cytokines often function in cascades, whereby one cytokine can increase or decrease the production of other downstream cytokines. The actions of a cytokine can thereby be indirect,

resulting in a biological action that would not be produced by the original cytokine itself. The classic example is the triumvirate of induced innate pro-inflammatory cytokines: IL-1β, TNF-α and IL-6. All three cytokines can be produced by distinct or the same cell types, and have a wide and somewhat overlapping range of target cells. IL-1β can induce expression of TNF-α and IL-6. TNF-α can induce expression of IL-1β and IL-6. IL-6, on the other hand, acts as a negative feedback, switching off IL-1β and TNF-α (Aderka *et al*., 1989; Hart *et al*., 1989). Other examples, discussed above, include the induction of IFN-γ by IL-12 and IL-18 to drive Th1 responses; the induction of IL-4, IL-5, IL-9, IL-13 and IL-19 by IL-4 to drive Th2 responses; and the induction of IL-17 by IL-23 to drive the development of Th17 cells. Finally, cytokines can also exert an effect on the production of other cytokines by either augmenting or reducing the level of expression of a receptor for the cytokine.

As more becomes known about the complex networks controlling cytokine interactions in biomedical mammalian species, more testable hypotheses will become apparent to understand similar networks in the chicken, with its reduced and different repertoire of cytokines.

CYTOKINE RECEPTORS

Cytokine receptors can be grouped into families based on common structural features, namely the class I (haematopoietin) cytokine receptor family, the class II (IFN/IL-10) cytokine receptor family, the TNFRSF, the IL-1 receptor family, the TGF-β receptor family and the chemokine receptors.

The largest family is the class I cytokine receptor family. Most of these receptors form heterodimers, but some are homodimers or heterotrimers. Many of these receptors form subfamilies, with one of the heterodimeric or heterotrimeric receptor chains, generally the signalling chain, being common to all members of the subfamily. For example, the IL-2 group of receptors (IL-2, IL-4, IL-7, IL-9, IL-15) share a common γ chain, the IL-6 group (IL-6, IL-11, CNTF, LIF, oncostatin M) a common gp130 chain, and the GM-CSF group (GM-CSF, IL-3, IL-5) a common β chain. The TNFRSF members are single chain receptors, thought to form homotrimers and become activated when cross-linked by their ligands, which are also homotrimers. The IL-1 family receptors (IL-1R and IL-18R) are heterodimers, formed of a cytokine-specific type I receptor and a receptor accessory protein (i.e. IL-1RI and IL-1RAcP, and IL-18RI and IL-18RAcP). Chemokine receptors are all seven-transmembrane-domain, G-protein-coupled receptors, and like their ligands, are subdivided into XCR, CCR, CXCR and CX3CR.

Class I Receptors

For the class I cytokine receptors in the chicken, all of the common signalling chains have either been cloned and characterized (common γ chain; Min *et al*., 2002a) and gp130 (Geissen *et al*., 1998) or are present in the chicken genome sequence (GM-CSFR β chain on chromosome 1).

For the IL-2 group of receptors, CD25 (IL-2R α chain) has been cloned and characterized (M. Iqbal, U. Pathania and P. Kaiser, unpublished results) and CD122 (IL-2R β chain) is present in the genome on chromosome 1 (Kaiser *et al*., 2005). The IL-15R α chain was cloned and characterized by Li *et al*. (2001). The gene for the IL-4R α chain is present in the genome on chromosome 14 but the IL-9R α chain gene is not identifiable in the genome. The IL-7R α chain has been cloned and characterized (L. Vervelde, personal communication).

For the gp130 group, the IL-6R α chain (Nishimichi *et al*., 2006) and the IL-11R α chain (Kawashima *et al*., 2005) have been cloned and characterized. For the GM-CSF group, CD116 (GM-CSFR α chain) and CD125 (IL-5R α chain) are present on chromosomes 1 and 5, respectively.

The other class I cytokine receptors (IL-12 and G-CSF) are present in the chicken. The two chains of the chicken IL-12R (IL12Rβ1 and IL12Rβ2) have been cloned and characterized (Balu, 2005). CD114 (G-CSFR) is present in the genome on chromosome 23.

Class II Receptors

The chicken IFN/IL-10 receptor gene cluster was cloned and characterized by Reboul *et al*. (1999). The cluster encodes both chains of the IFN-α/β receptor, one chain of the IFN-γ

receptor (IFNGR2) and one chain of the IL-10R (IL10R2). CD119 (IFNGR1) is encoded on chromosome 3. The gene encoding the second chain of the IL-10R (IL10R1) is not present in the genome sequence.

TNFRSF

The chicken TNFRSF members are discussed above.

IL-1 Family Receptors

The chicken type I IL-1R was the first chicken cytokine receptor to be cloned and characterized (Guida *et al*., 1992). Soluble type I IL-1R blocks chicken IL-1 activity (Klasing and Peng, 2001). The gene encoding chicken IL-1RAcP lies on chromosome 9. The genes encoding chicken IL-18RI and IL-18RAcP both lie on chromosome 1.

TGF-β Family Receptors

None of the chicken TGF-β receptors have been characterized. However, the genes for TGF-βRI (chromosome 2), TGF-βRII (chromosome 2) and TGF-βRIII (chromosome 8) are all present in the genome.

Chemokine Receptors

Genes for XCR and CX3CR are present in the chicken genome (Kaiser *et al*., 2005). Of the 10 CCR genes so far identified in human and mouse, direct orthologues of only 5 (CCR4, CCR6, CCR7, CCR8 and CCR9) can be identified in the chicken genome. In human and mouse, several CCR (including CCR1–3 and CCR5, the receptors for the MIP family chemokines) cluster together on chromosomes 3 and 9, respectively. In the equivalent region of the chicken genome there are three CCR genes. Two of these branch together with human and mouse CCR1, CCR2, CCR3 and CCR5 in phylogenetic analysis. The third branches with chicken, human and mouse CCR8, in mammals the receptor for CCL1, an MCP family chemokine. Pending further functional studies on these chicken CCR, we proposed that they should be named chCCRa–c, respectively (Kaiser *et al*., 2005). Of the six CXCR identified to date in man and mouse, only three (CXCR1, CXCR4 and CXCR5) can be identified in the chicken (Kaiser *et al*., 2005).

REAGENTS AVAILABLE

The availability of reagents to avian cytokines is described in more detail in Appendix 2. Real-time quantitative RT-PCR assays, to measure cytokine expression at the mRNA level, have been described for a wide panel of chicken cytokines (e.g. Eldaghayes *et al*., 2006), and are simple assays to design and optimize. Polyclonal antisera have been raised to several cytokines, and some of these are commercially available (e.g. Serotec supply pAb to IFN-α, IFN-β, IFN-γ, IL-1β and IL-6). Similarly, few mAbs have been described to chicken cytokines or their receptors. There are mAbs to IL-2 (Miyamoto *et al*., 2001; Rothwell *et al*., 2001), IL-6 (Nishimichi *et al*., 2005), IL-15 (Min *et al*., 2002), IFN-α (Serotec, unpublished), IFN-γ (Lambrecht *et al*., 2000; Yun *et al*., 2000) and IL-15R α chain (Li *et al*., 2001). As part of the UK BBSRC Immunological Toolbox Programme, mAbs have also been raised to IL-4, IL-6, IL-10, IL-12β (which also recognizes IL-12 p70), IL-19 and IL-22 (M. Iqbal, S. Balu, U. Pathania and P. Kaiser, unpublished results).

ASSAY SYSTEMS AVAILABLE

Many of the publications describing the cloning and characterization of a chicken cytokine have described its biological activity using a bioassay. These include bioassays for IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, IL-13, IL-15, IL-16, IL-17, IL-19, IL-22, IFN-α/β, IFN-γ, TGF-β, GM-CSF,

BAFF and several chemokines. The main problem with bioassays is specificity, or rather the lack of it. For example, the bioassay used to measure IL-1β activity (proliferation of thymocytes co-stimulated with sub-optimal levels of mitogen and IL-1β) will also measure IL-2 (Lawson *et al*., 2000). Enzyme-linked immunosorbent assays (ELISA) have the advantage of specificity, but of course also measure protein that may not be bioactive. There are very few available ELISA for chicken cytokines. There is a commercial capture ELISA for IFN-γ (Lambrecht *et al*., 2000), and published ELISA for IL-2 (Miyamoto *et al*., 2001; Rothwell *et al*., 2001) and IL-15 (Min *et al*., 2002b). ELISA for other cytokines are in development. More sophisticated cytokine expression assays, such as enzyme-linked immunospot (ELISPOT) or cytometric bead assays, are yet to be developed for the chicken, with the exception of an ELISPOT for IFN-γ (L. Vervelde and M. Arianns, personal communication).

REGULATION OF CYTOKINE RESPONSES

The powerful biological activities of cytokines are potentially harmful to the organism. It is thus perhaps not surprising that various mechanisms are in place to counteract overshooting cytokine responses. These include cytokine-inducible synthesis of negative regulatory factors such as suppressor of cytokine signalling (SOCS) (Ilangumaran *et al*., 2004; Leroith and Nissley, 2005) and protein inhibitor of activated STAT (PIAS) proteins (Shuai and Liu, 2005), which silence cytokine gene expression and thus terminate the immune response. Experiments with knockout mice showed that lack of these negative regulators can lead to chronic inflammation and disease (Leroith and Nissley, 2005; Shuai and Liu, 2005). No avian homologues of mammalian SOCS and PIAS have to date been characterized.

Soluble cytokine-binding proteins represent an alternative strategy for protection against overshooting cytokine responses (Levine, 2004). The biological effects of TNF-α, IL-1β, IL-6, IL-18 and IFN-γ are regulated in this manner (Novick and Rubinstein, 2004). Binding of cytokines by soluble receptors usually reduces their biological effect. Unlike their membrane-bound counterparts, soluble cytokine receptors are not physically linked to molecules that could generate and transmit stimulatory signals. Soluble cytokine-binding proteins thus usually serve as cytokine antagonists. Interestingly, however, soluble receptors can also serve to prolong and even amplify cytokine signals as demonstrated in the case of the soluble IL-6 receptor/IL-6 complex (Rose-John and Neurath, 2004). No avian homologues of mammalian cytokine-binding proteins have to date been characterized.

VIRAL PROTEINS BLOCKING CYTOKINE ACTION

Poxviruses have evolved successful strategies to evade the host immune response that are based on soluble cytokine-binding proteins (discussed in Chapter 16). Poxvirus-encoded proteins were described that efficiently bind and neutralize TNF-α, IFN-γ and IFN-α/β (Alcami and Smith, 1996). Infection studies with mutant viruses lacking these factors demonstrated their role as virulence-determining factors in immunocompetent hosts (McFadden *et al*., 1995).

Avian poxviruses are poorly characterized in this regard. Their large genomes contain many genes which code for proteins of unknown function. Recently, the product of one such gene, ORF016 of fowlpox virus, was shown to bind specifically to chicken IFN-γ (Puehler *et al*., 2003). The ORF016 protein of fowlpox virus lacks all features of known cellular IFN-γ receptors or antagonistic IFN-γ-binding proteins of other poxviruses. *In vivo* evidence that fowlpox virus uses the ORF016 gene product to counteract the antiviral immune response of chickens is still missing.

Two groups independently predicted that two different fowlpox virus genes (fpv073 and fpv214) would encode candidate IL-18-binding proteins (Afonso *et al*., 2000; Laidlaw and Skinner, 2004). Only one of the predicted genes (fpv214) encoded a protein with a conserved IL-18-binding motif and, interestingly, knockout of this gene in a recombinant fowlpox

virus expressing IBDV VP2 resulted in an enhanced cell-mediated immune (CMI) response against IBDV when chickens vaccinated with the recombinant were challenged with IBDV. The enhancement was comparable to, but less dramatic than, the enhancement observed when the VP2 recombinant fowlpox virus co-expressed chicken IL-18, whether fpv214 was intact or not (Eldaghayes, 2005).

Viral evasion strategies that target the action of cytokines are not limited to cytokine neutralization with the help of soluble receptors (see also Chapter 16). Most successful RNA viruses seem to encode virulence factors that target cellular factors involved in either expression of type I IFN genes or signalling of IFN in target cells (reviewed in Garcia-Sastre and Biron, 2006; Haller *et al*., 2006). Here, the discussion will be limited to two viruses that are important poultry pathogens, namely influenza A virus (FLUAV) and NDV. The non-structural protein NS1 of FLUAV is an auxiliary factor which seems to play a crucial role in inhibiting type I IFN-mediated antiviral responses of the host. This conclusion was most clearly illustrated with knockout mice lacking important components of the type I IFN system such as STAT1 or protein kinase receptor (PKR). Unlike wild-type mice, which manage to efficiently control infections with FLUAV mutants lacking NS1, STAT1- or PKR-deficient mice developed severe disease after challenge with NS1-deficient FLUAV (Garcia-Sastre *et al*., 1998; Bergmann *et al*., 2000). The mode of NS1 action is complex (Krug *et al*., 2003) and seems to result in decreased efficacy of pathogen recognition by the infected host (Garcia-Sastre *et al*., 1998), as well as in decreased IFN-mediated antiviral response of the host cell (Seo *et al*., 2002; Hayman *et al*., 2006; Min and Krug, 2006). NS1 prevents activation of latent IFN-regulatory factor 3 and synthesis of IFN in the infected host cells, at least in part by sequestering viral double-stranded RNA and preventing stimulation of cellular sensors of double-stranded RNA, such as RIG-I and mda-5 (Kato *et al*., 2005, 2006).

The NS1 protein also appears to determine the replication efficacy of FLUAV in avian cells by counteracting the host IFN response. This view is based on the observation that unlike wild-type FLUAV, an NS1-deficient mutant virus which grew reasonably well in very young (6-day-old) embryonated chicken eggs did not grow substantially in older (11-day-old) embryonated chicken eggs (Talon *et al*., 2000). The latter embryos are believed to possess a more mature innate immune system that responds more vigorously to viral stimuli. Intriguingly, C-terminal truncations of NS1 were observed in natural isolates of H7N1 FLUAV circulating in poultry (Dundon *et al*., 2006), and a polymorphism in a domain near the C-terminus of NS1 was suggested to influence the pathogenicity of avian H5N1 FLUAV in mammals (Obenauer *et al*., 2006). However, the ultimate proof that NS1 also determines virulence of avian FLUAV in their natural hosts is still lacking. It should be noted that avian FLUAV strains seem to possess additional factors of unknown identity that can suppress induction of IFN by infected host cells (Marcus *et al*., 2005).

NDV uses an auxiliary protein, designated V, to counteract the host immune response. Like V proteins of other paramyxoviruses, the V protein of NDV is believed to block IFN-induced antiviral responses by targeting STAT proteins for proteasome-mediated degradation (Andrejeva *et al*., 2002). Mutant NDV lacking the ability to synthesize V were reported to grow poorly in 10–14-day-old embryonated chicken eggs and chicken embryo fibroblasts compared to wild-type virus (Huang *et al*., 2003; Park *et al*., 2003). *In vivo* studies with mutant NDV lacking V demonstrated severe attenuation in 1-day-old chicks and a complete inability to cause disease in 6-week-old chickens (Huang *et al*., 2003).

POTENTIAL USE OF CYTOKINES AS VACCINE ADJUVANTS

Since endogenous cytokines are potent regulators of immunological reactions, it is reasonable to believe that vaccine formulations which include exogenous cytokines might yield enhanced immune responses (Nash *et al*., 1993; Pardoll, 1995). A comprehensive review summarizing recent work aimed at enhancing the immune response of chickens towards standard vaccines

has recently been published (Asif *et al*., 2004). Most studies in chickens demonstrated beneficial effects of several cytokines, including IL-1β, IL-2, MGF, G-CSF, IFN-α and IFN-γ (Lowenthal *et al*., 1998; Schijns *et al*., 2000; Min *et al*., 2001; Djeraba *et al*., 2002; Hilton *et al*., 2002b). A more recent study further demonstrated that chicken IL-18 can enhance the humoral immune response to vaccine antigens of different origin (W.G. Degen *et al*., 2005). However, although the adjuvant effect of cytokines was significant in most of these studies, a wide use of cytokines in poultry vaccines is not evident mainly because of high production costs and additional practical hurdles. Co-expression of cytokine genes in fowlpox vaccine strains or other viral vectors might overcome most of these problems (Karaca *et al*., 1998), but may also raise important safety concerns (Mullbacher and Lobigs, 2001).

IMPROVED VACCINES BASED ON VIRAL MUTANTS LACKING CYTOKINE ANTAGONISTS

A new, safe and inexpensive way to use the immunostimulatory effects of cytokines is emerging. From the knowledge discussed above that most RNA viruses code for proteins with IFN antagonistic activity, it should be possible to design viral vectors that strongly induce endogenous cytokine gene expression in the vaccinated host (Palese *et al*., 1999). Talon *et al*. (2000) recently evaluated this idea using FLUAV lacking the IFN antagonist NS1. They found that although completely avirulent if used as a vaccine at a high dose, NS1-deficient FLUAV-induced solid protection in mice against subsequent challenge with a highly lethal FLUAV strain. It remains to be determined whether NS1-deficient FLUAV strains can be developed into a product that can be used for testing in animals that are natural hosts of FLUAV, such as pigs, horses or poultry.

Mebatsion *et al*. (2001) determined whether NDV lacking the IFN antagonist factor V might qualify as a live vaccine against Newcastle disease in chickens. Since the genetic information for the V protein is derived by editing from a bi-cistronic viral gene, viable NDV mutants with complete V gene deletions cannot be generated. Therefore, a V-deficient mutant virus with an altered editing site was used for this study. This candidate vaccine strain was completely apathogenic when injected into 18-day-old chicken embryos, although it induced remarkably good protection against challenge with virulent NDV at 2 weeks of age (Mebatsion *et al*., 2001). Interestingly, additional work showed that this virus is genetically unstable and may quickly regain replication competence and pathogenicity (Mebatsion *et al*., 2003), highlighting an important difficulty of this vaccine approach in NDV and other viruses in which the IFN antagonistic factor is not encoded by a non-essential gene. To develop V-deficient NDV into a safe chicken vaccine, additional mutations might need to be introduced into the viral genome.

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11 IMMUNOGENETICS AND MAPPING IMMUNOLOGICAL FUNCTIONS

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INTRODUCTION

Proper function of the immune system is essential to maintain health in avian species, and the genetics of a bird defines its maximum achievable immune responses. A comprehensive disease control programme contains many components, including biosecurity, sanitation, vaccination and host genetics. Genetic enhancement of immunity has permanent and cumulative effects in a breeding population, and is therefore an environmentally friendly approach to maintaining health in poultry (Lamont, 2004, 2006).

Microorganisms with asymptomatic presence in poultry may still be food-borne pathogens that cause disease in humans upon consumption of improperly prepared poultry products (Doyle and Erickson, 2006). Therefore, reduction of the microbial burden in poultry is an important step in pre-harvest food safety. The identification of biomarkers associated with immune function can be effectively used to enhance host resistance to pathogens and thereby reduce disease and pharmaceutical treatments in the live bird and microbial contamination of poultry products (Lamont, 1998).

In addition to maintaining health, some genes that are typically understood as having a primary function in immunity are also associated with production traits such as growth (Ye *et al.*, 2006). The health and production traits may be positively correlated, such as the situation in which greater health levels support more vigorous growth. Conversely, they may be negatively correlated, which may be the result of resource allocation; e.g., animals that are genetically

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predisposed to allocate their protein resources to muscle growth may direct fewer resources towards antibody-mediated immunity (Pinard-van der Laan *et al*., 1998).

THE CHICKEN GENOME

The recent availability of the chicken genome draft sequence (Hillier *et al*., 2004) and the accompanying single-nucleotide polymorphism (SNP) map (Wong *et al*., 2004) has opened a new era for research and for the understanding of how genetic variation can impact on immune response, health and response to pathogens. This, in turn, will enable more informed and effective use of genetic enhancement strategies as means to protect avian health through genetic selection for improved immune responsiveness, the use of recombinant proteins to enhance immunity and vaccine efficacy, and production of specific pathogen-resistant transgenic lines of birds.

The chicken genome has many features that are unique from those of mammals (Burt, 2005). The chicken genome is approximately one-third the size of that in mammals. Birds accommodate this reduced genome size in part by a greatly reduced content of repetitive elements, which represent about 10–12% of the chicken genome in contrast to almost 50% of the typical mammalian genome. The compact genome of the chicken also has a much higher crossover rate than that seen in mammals, ranging from an average of 2.8–6.4 cM/Mb in macrochromosomes and microchromosomes, respectively. Humans average 1–2 cM/Mb.

The chicken genome sequence has served as a platform for gene discovery and analysis. Unfortunate for those interested in chicken immunogenetics is the finding that the major histocompatibility complex (MHC), with its dense gene content and many duplicated genes, is poorly represented in the first draft genome sequence. However, this region was already the subject of genomic investigation before the complete draft sequence was produced (Kaufman *et al*., 1999) and is currently receiving intense study to resolve the shortfalls of the first draft sequence. The identification of many other genes of the immune system was initially hampered by their rapid evolutionary rate and limited genomic sequence similarity to mammalian sequences (Staeheli *et al*., 2001). However, analysis of large expressed sequence tag (EST) sets identified almost 200 putative immunity-related gene sequences (Smith *et al*., 2004). Use of the genome sequence, especially in a species-comparative manner, has also contributed much to the identification of cytokines and their receptors (Kaiser *et al*., 2005). For most of the genes identified through *in silico* (bioinformatic) methods, the task still remains to demonstrate their unique role(s) in avian immune responses through functional genomic analysis and immunological assessment.

SELECTING FOR IMMUNOLOGICAL TRAITS IN THE CHICKEN

Selection for immunological traits in the chicken has been conducted with the immediate aim of understanding the genetic control of immune responses and the overall goal of enhancing flock health. Once biomarkers associated with immune responses are identified, they can be used in breeding programmes to enhance health. The biomarkers may be of many different types. Early studies were limited to easily assess serological and other markers, such as serum antibodies and cell-surface antigens. These biomarker types are still relevant because of the ease and relative economy of their measurement. Within the past two decades, however, the advent of molecular genetics and its application in studies on farm animals has opened the door to DNA marker identification. Rapidly progressing research into gene expression in avian species will likely define expression-based biomarkers for immunity and disease resistance, and is already being incorporated into integrated studies to identify genes controlling host resistance to Marek's disease (Liu *et al*., 2001).

Although it is feasible to genetically select for host responses to individual pathogens (Beaumont *et al*., 2003; Bumstead, 2003), this approach is less frequently applied than selection for immune response. The cost of pathogen-challenge trials is very high and presents a

biosecurity risk. Additionally, it is difficult to predict the specific type and range of pathogens that commercial poultry may encounter in the field, thus complicating the decision about which pathogens should be considered in genetic selection of breeding stock. More attention, therefore, has been directed to elucidating the genetic control of immune responses, with the underlying assumption that immune responses will provide protection against narrow or broad ranges of pathogens. The methodology for identification of genes or markers associated with immune responses and with disease resistance is fundamentally the same.

Selection for immune response in chickens has most frequently been done based on measurement of antibody levels to a defined antigen at a specific age. It is important, however, to keep in mind that, although the genetic selection may be based on a single trait, the correlated responses in the complex immune system may simultaneously alter many immune responses. Several large, long-term studies have been conducted, and collectively have generated much information about the genetics of antibody response, as well as the correlated changes that accompany selection for antibody response (reviewed in Lamont *et al*., 2003). Two major experiments, at Virginia Tech (USA) and at Wageningen University (The Netherlands), have implemented long-term, divergent, single-trait selection for antibody response to sheep red blood cells (SRBC) as the stimulating antigen. Although clearly not an infectious agent, SRBC have the advantage that their antibodies are easily and economically measured with haemagglutination assays. Another experiment (Hebrew University, Israel) has selected for antibody response to *Escherichia coli* vaccine, thus assessing response to pathogen-derived antigens. Other experiments have been designed to reflect the complexity of the total immune response by incorporating multiple immune-response traits, either combined in a single selection index (Iowa State University, USA) or selected individually in separate selection lines (INRA, France). Major lessons emerging from these studies are described below.

The heritabilities of the measured immune-response traits indicate that genetic selection is quite feasible, especially for antibody levels for which the heritabilities are moderate, generally around 0.2–0.3 (Siegel and Gross, 1980; Cheng *et al*., 1991; Leitner *et al*., 1992; Pinard *et al*., 1992). For the non-antibody traits, such as the cell-mediated response to phytohaemagglutinin or the phagocytic response measured by carbon clearance, the estimated heritabilities were lower (0.05–0.15), suggesting that these traits would be more difficult to alter by genetic selection (Cheng *et al*., 1991; Lamont *et al*., 2003). The genetic correlations among the various types of immunity measures were generally low, which indicates that each is independently controlled and therefore can be independently altered by genetic selection.

Correlated changes that occur concomitantly with genetic selection for immunological traits are important to consider within the context of the overall performance of the flock. Selection for immune response is conducted with the assumption that it will alter (improve) resistance to disease. This was, indeed, verified by empirical testing of the immune-response selected lines, and the divergent lines generally exhibited divergent resistance properties to several diseases (Lamont *et al*., 2003). A cautionary note, however, comes from the examination of the Virginia Tech lines, in that the high antibody line was relatively more resistant than the low antibody line to most, but not to all, pathogens (Gross *et al*., 1980; Martin *et al*., 1986; Dunnington *et al*., 1991). The specific relationship (beneficial or detrimental) of immune-response traits to resistance to specific pathogens may vary, and must be defined before undertaking a genetic selection programme with the intent to improve disease resistance.

Changes in allele frequencies correlated with immune-response selection provide suggestive evidence that the gene is associated with the selected trait, and therefore defines a candidate gene for more intensive study to verify its role in immunity. Within the aforementioned selection experiments, marked alterations in the MHC genes were consistently evident, although the fraction of phenotypic variation explained was not large, suggesting that the selected immune trait was under polygenic control (Dunnington *et al*., 1984; Pinard and van der Zijpp, 1993; Kean *et al*., 1994; Yonash *et al*., 1999b).

To be effectively implemented in a breeding programme to improve bird health, genetic selection for immune-response traits must not unduly compromise other important traits. In

this context, the relationship between antibody response and growth is important to consider. In both of the single-trait selection experiments for antibodies to SRBC, there was a negative correlation between antibody level and body weight or growth (Lamont *et al.*, 2003). This negative correlation is postulated to arise from competition for limited nutrients among the different physiological systems, such as protein deposition in muscle and secretion in antibodies (Pinard-van der Laan *et al.*, 1998). However, the benefit or detriment of genetic enhancement of antibody production on growth might differ between high-hygiene environments (such as breeding companies and research facilities) and low-hygiene environments with significant environmental antigen challenges, such as might be found in typical commercial production. There is evidence for this type of environmental interaction with genetic control of production traits for several genes of the immune system (Ye *et al.*, 2006). The "cost" of genetically programming the animal for high antibody production might be beneficial in a low-hygiene environment in which protection against pathogens is essential to maintain health. The specifics of the nutrient requirements of an activated immune system, and the details of the genetic correlations between immune and production traits remain to be elucidated.

KEY GENE LOCI FOR IMMUNOLOGICAL TRAITS

Although most immunological traits are polygenic traits controlled by multiple genetic loci (quantitative trait loci, QTL), there are many individual genes and families of genes that have major effects on immune response and/or response to pathogens in avian species. The best known of these key gene loci is the MHC, which was initially characterized as a blood group antigen locus (Briles *et al.*, 1950) and thereafter demonstrated to be the region containing those genes controlling tissue histocompatibility (Schierman and Nordskog, 1961). The chicken MHC represents the longest-term application of marker-assisted selection (MAS) in animal production, with decades of commercial breeder selection in layer chickens for B blood group antigens linked to the MHC alleles that confer resistance to Marek's disease. After its identification in the chicken, associations of the MHC with immunity and disease resistance in many other domestic animal species have been demonstrated (Schook and Lamont, 1996; Rothschild *et al.*, 2000).

The avian MHC is described in detail in Chapter 8, but it is worthwhile noting here that the chicken MHC is considered to be "minimal essential" because of the compact and gene-dense nature of its genomic organization (Kaufman *et al.*, 1999). The plethora of immune-related genes that are located within the MHC may explain the varied and pleiotropic immune responses that are reported to be associated with this region. The high SNP frequency of the MHC-bearing chromosome 16, in comparison to the genome as a whole (Wong *et al.*, 2004), is also understood as a mechanism for the MHC genes to encode highly polymorphic proteins that allow a wide repertoire of antigen recognition and cellular interaction.

The immunoglobulin (Ig) genes encode non-specific and specific antibodies, which are crucial for humoral immune defence against many types of pathogens (see Chapter 6). Because antibodies are easily measured and highly heritable, they have long been the subject of immunogenetic studies, which have clearly demonstrated the ability to alter the level, kinetics and persistence of antibody production, as described earlier in this chapter. Genetic selection to modulate antibody production (which presumably acts primarily through genes that modulate the expression of Ig genes) can enhance poultry populations in many different ways. These alterations can include more efficacious response to vaccination, more rapid production, higher levels, greater affinity and/or more persistent production of specific antibodies. It is also of interest to define the genetics controlling deposition of maternal antibodies into the egg, both for protection of offspring via passive antibody (or conversely, interference with vaccination), and to identify those birds that will deposit large quantities of Ig into the egg for use of hens in production of recombinant proteins.

The CD antigens comprise a large family of cell-surface proteins serving diverse roles in immune responsiveness, with most CD antigens being limited to expression on specific cell

types and/or differentiation stages (see Chapter 5). Recent characterization of two CD1 genes within the chicken MHC region suggests an evolutionary origin of these genes by extensive sequence divergence from the classical MHC genes, while still being genetically linked to the MHC (Miller *et al*., 2005).

Cytokines, chemokines and their receptors serve as an extensive and redundant system to ensure effective intercellular communication and coordination in the immune system. Most avian cytokine and chemokine genes have only recently been described (De Vries *et al*., 2005; Kaiser *et al*., 2005; Chapter 10). Identification of the cytokine and cytokine receptor genes, and production of the recombinant proteins, opens possibilities to genetically select breeding populations for more effective cytokine responses and to use cytokines as vaccine adjuvants (Asif *et al*., 2004).

More recently, additional genes of the innate immune response have been actively characterized in chickens. The Toll-like receptors (TLR) serve as cell-surface receptors, with the different TLR being relatively specific for various classes of antigens. Members of the TLR are described in detail in Chapter 7. The avian defensins, or gallinacins, are small antimicrobial peptides, which have a broad range of activity against Gram-negative and Gram-positive bacteria. Genetic variation in chicken gallinacin genes has been demonstrated to be associated with antibody titre to *Salmonella enteritidis* vaccine (Hasenstein *et al*., 2006). Once thought to be relatively passive, the important role of the innate immune system as a first line of defence, as well as a key component in orchestration of an effective acquired immune response, is now becoming more fully appreciated.

The Mx gene is of special interest for its potential role in genetic resistance to avian influenza virus infection, based on activity against influenza virus in mice (Staeheli *et al*., 1986). In transfected cell lines, mutation at amino acid position 631 in the carboxy terminus of the chicken Mx gene was demonstrated to be essential in determining the antiviral specificity of the Mx protein against vesicular stomatitis virus (Ko *et al*., 2004). Identification of this mutation within commercial breeding stocks would allow selection for changes in allelic frequency to increase the resistance level of the population to avian influenza. Skewed allele frequencies of a favourable allele in native Chinese breeds, compared to commercial populations, suggests that this allele may respond to natural selection pressure from the environment, as well as having negative correlations with production traits (Li *et al*., 2006).

Many additional gene loci that are not currently identified as biological candidate genes for immune function are likely to be identified in the near future, as studies expand to investigate genes in a more global fashion and are not limited to the "known" immune function genes. These studies include such approaches as profiling gene expression with multi-tissue microarrays and using genome-wide panels to evaluate SNP in coding regions, or combinations of these techniques (de Koning *et al*., 2005).

STATISTICAL APPROACHES TO DETECT CHROMOSOMAL REGIONS THAT CONTROL IMMUNOLOGICAL TRAITS AND DISEASE RESISTANCE

Over the past decades, many experimental approaches have been developed for the use of anonymous genetic markers to identify chromosomal regions that harbour genes (QTL) that control quantitative traits (Andersson, 2001). These same methods can also be used to identify genes or chromosomal regions that control immunological traits and disease resistance (Cheng, 2003). All methods rely on identifying statistical associations of genotype at marker loci with phenotype, e.g. by contrasting the mean phenotype of individuals that have alternate marker genotypes. A difference in mean phenotype (e.g. pathology score or mortality) indicates that the marker is linked to a QTL or, in the ideal case, represents the causative mutation for the QTL (direct marker). However, this does not mean that every marker which is linked to a QTL is expected to show a mean difference in phenotype; besides linkage, the second condition that is needed to create a difference in mean phenotype between alternate marker genotypes is presence of linkage disequilibrium (LD) between the marker and the QTL. The concept of LD is important

for both QTL detection and the use of these QTL in genetic selection (MAS), and will be explained next.

Linkage Disequilibrium

Consider a marker locus with alleles *M* and *m* and a linked QTL with alleles *Q* and *q*. Alleles at the two loci are arranged in haplotypes on the two chromosomes of a homologous pair that each individual carries. For example, an individual with genotype *MmQq* could have the following two haplotypes: *MQ/mq*, where the / separates the two homologous chromosomes. Alternatively, it could have the following marker-QTL linkage phases: *Mq/mQ*. The arrangement of alleles in haplotypes is important because progeny inherit one of the two haplotypes that a parent carries, barring recombination.

Presence of LD relates to the relative frequencies of alternative haplotypes in a population. In a population that is in linkage equilibrium (LE), alleles at two loci are randomly assorted into haplotypes, i.e. chromosomes or haplotypes that carry marker allele *M* are no more likely to carry QTL allele *Q* than are chromosomes that carry marker allele *m*. In this case, there is no value in knowing an individual's marker genotype because it provides no information on QTL genotype. If the marker and QTL are in LD, however, there will be a difference in the probability of carrying *Q* between chromosomes that carry *M* and *m* marker alleles and, therefore, we would also expect a difference in mean phenotype between marker genotypes. This situation would make the marker an effective predictor of the phenotype associated with the QTL.

The main factors that create LD in a population are mutation, selection, drift (inbreeding), and migration or crossing (e.g. Goddard and Meuwissen, 2005). The main factor that breaks down LD is recombination, which can rearrange haplotypes that exist within a parent in every generation. The rate of decay of LD depends on the rate of recombination between the loci, i.e. on their genetic distance on the chromosome; for tightly linked loci, any LD that has been created will persist over many generations but for loosely linked loci ($r > 0.1$), LD will decline rapidly over generations. Some regions of the genome, especially those containing similar genes in tandem, as exists for several families of immune-related genes, are more prone to recombination. This is seen in, for example, the microchromosome bearing the MHC (Hillier *et al*., 2004).

Although a marker and a linked QTL may not be in LD across the population, LD will always exist within an individual family, even between loosely linked loci. This fact can be effectively used to design and analyse studies to identify marker-QTL associations. Consider a double heterozygous sire with haplotypes *MQ/mq*. The genotype of this sire is identical to that of an F_1 from inbred lines. This sire will produce four types of gametes: non-recombinants *MQ* and *mq* and recombinants *Mq* and *mQ*. Because the non-recombinants will have higher frequency, depending on recombination rate, this sire will produce gametes that will be in LD. This LD will extend over larger distance, because it has undergone only one generation of recombination. This specific type of LD, however, only exists within this family; progeny from another sire, e.g. an *Mq/mQ* sire, will also show LD, but the LD is in the opposite direction because of the different marker-QTL linkage phase. On the other hand, *MQ/mQ* and *Mq/mq* sire families will not be in LD because the QTL does not segregate in these families. When pooled across families these four types of LD cancel each other out, resulting in LE across the population. Nevertheless, the within-family LD can be used to detect QTL and for MAS, provided the differences in linkage phase are taken into account in the experimental design and statistical analyses.

Experimental Designs to Detect QTL

Using the alternate types of LD described above, the main experimental strategies that have been used to detect QTL in domestic animal populations are summarized in Table 11.1 and are described further in the following sections (see also Andersson, 2001; Andersson and Georges, 2004).

TABLE 11.1 Summary of Strategies for QTL Detection

Type of Population	Line or Breed Crosses		Outbred Population			
	F_2 or Backcross	Advanced Intercross	Half- or Full-Sib Families	Extended Pedigree	Non-pedigreed Population Sample	
Type of markers	LD[a]-markers		LE[b]-markers		LD-markers	
Genome coverage	Genome-wide		Genome-wide		Candidate gene regions	Genome-wide
Marker density	Sparse	Denser	Sparse	Denser	Few loci	Denser
Type of LD used	Population-wide LD		Within-family LD		Population-wide LD	
Number of generations of recombination used for mapping	1	>1	1	>1	>>1	
Extent of LD around QTL	Long	Smaller	Long	Smaller	Small	
Map resolution	Poor	Better	Poor	Better	High	

[a]Linkage disequilibrium.
[b]Linkage equilibrium.

Line or Breed Crosses

Crossing two lines or breeds that differ in gene and, therefore, haplotype frequencies, creates extensive LD in the F_1 generation and much of this LD will still extend over large distances in the F_2 generation because it has undergone only one generation of recombination. This enables detection of QTL across the genome that differ in frequency between the two breeds with only a limited number of markers spread over the genome (~every 15–20 cM). This has formed the basis for the extensive use of F_2 or backcrosses between breeds or lines for QTL detection (for a review, Andersson, 2001; also Zhou *et al*., 2003; McElroy *et al*., 2006). This extensive LD enables detection of QTL that are some distance from the markers but also limits the accuracy with which the position of the QTL can be determined. The latter can be overcome by using advanced intercrosses (e.g. F_6 or higher). In such populations, the extent of LD at larger distances has been further eroded by repeated recombination and will, therefore, span much shorter distances around a QTL. Detection of QTL in advanced intercrosses requires a denser marker map but also enables more precise positioning of the QTL (Darvasi and Soller, 1995).

Within-Family LD in Outbred Populations

Because linkage phases between the marker and QTL can differ from family to family, use of within-family LD for QTL detection requires marker effects to be fitted on a within-family basis, rather than across the population. Similar to F_2 or backcrosses, however, the extent of within-family LD is extensive and, therefore, genome-wide coverage is provided by a limited number of markers. However, similar to F_2 crosses, significant markers may be some distance from the QTL. Many examples of successful applications of this methodology for detection of QTL for immune response, disease resistance and other traits in poultry are available in the literature (for a review, see Hocking, 2005).

Population-Wide LD in Outbred Populations

The amount and extent of LD that exist in outbred populations which are used for research and/or for genetic improvement is the net result of all forces that create and break-down LD and is, therefore, the result of the breeding and selection history of each population, along with random sampling. On this basis, populations that have been closed for many generations are expected to be in LE, except at closely linked loci. Therefore, in those populations, only markers that are tightly linked to QTL may show an association with phenotype, and even then

there is no guarantee of presence of sufficient LD between the marker and the QTL, because of the chance effects of random sampling. These closed populations are typical of the non-inbred lines kept for experimental studies at research institutions.

There are two strategies to find markers in such populations that are in population-wide LD using QTL:

1. Evaluating markers that are in, or close to, genes that are hypothesized to be associated with the trait of interest (candidate genes).
2. A genome scan using a high-density marker map, with a marker every 0.5–2 cM.

The success of both these approaches obviously depends on the extent of LD in the population. Studies in human populations have generally found that LD extends over much less than 1 cM. Thus, many markers are needed to obtain sufficient marker coverage to enable detection of QTL based on population-wide LD. Opportunities to utilize population-wide LD to detect QTL in avian populations may be considerably greater because of the effects of selection and inbreeding. Indeed, several studies, including some in poultry (Heifetz *et al*., 2005), have identified substantial LD in domestic animal populations. This is advantageous for QTL detection, but disadvantageous for identifying the specific causative mutations of these QTL. With extensive LD, markers that are some distance from the causative mutation can show an association with phenotype (Andersson and Georges, 2004).

The candidate gene approach utilizes knowledge from species that are rich in genome information (e.g. human, mouse), effects of mutations in other species, previously identified QTL regions, and/or knowledge of the physiological basis of traits to identify genes that are likely to play a role in the physiology of the trait (Rothschild and Soller, 1997). After mapping and identification of polymorphisms, the association of genotype at the candidate gene with disease or immune phenotype can be estimated in a closed breeding population.

Recent advances in genome technology have enabled sequencing of entire genomes, including those of several livestock species. In addition, sequencing has been used to identify large numbers of positions in the genome that include SNP. For example, in the chicken over 2.8 million SNP have been identified by comparing the sequence of the red jungle fowl to that of three domesticated breeds (Wong *et al*., 2004). This, combined with reducing costs of genotyping, now enables detection and fine-mapping of QTL using LD-mapping with high-density marker maps (Meuwissen and Goddard, 2000; Andersson and Georges, 2004).

Statistical Procedures for QTL Detection

As previously mentioned, QTL detection is based on the identification of statistical associations between phenotype and the inheritance or presence of specific chromosomal regions. Several statistical methods have been developed to detect QTL within the context of the experimental designs described above (Weller, 2001). These range from methods based on least squares regression, to variance component mixed model approaches, and complex segregation analysis methods using maximum likelihood or Bayesian approaches. The use of marker genotype information in these models ranges from simply fitting marker genotypes as fixed effects in the model of analysis, to fitting probabilities of inheritance of specific parental alleles at putative positions on the chromosome for the QTL that are derived from marker data, as in least squares regression interval mapping, to the use of marker genotypes to quantify covariances between putative QTL effects of pairs of individuals based on identity by descent probabilities derived from marker genotypes.

For most quantitative traits, methods derived from standard statistical methods that are based on a normal distribution of phenotypes can be used. Many traits associated with disease do, however, not follow a normal distribution but are recorded on a binary scale (e.g. presence/absence of pathogen or pathology) or survival scale (e.g. age at mortality). Although linear models can in principle be applied for the analysis of phenotypes that do not follow a normal distribution, more appropriate statistical methods are available for the analysis of such data and these can also be applied to analysis of marker and phenotypic data for the purposes of

QTL detection (Rebai, 1997). Examples of such applications include the use of survival analysis methods (Moreno *et al*., 2005; McElroy *et al*., 2006) and use of logistic regression methods for analysis of disease incidence data (Kadarmideen *et al*., 2000).

Immune Response QTL

To date, most of the identified immune-response QTL have been for antibody responses, because of the ease of measurement of this trait, moderate heritability and the availability of divergently selected lines to form appropriate resource populations for study. QTL have been associated with antibody production to SRBC (Siwek *et al*., 2003; Zhou *et al*., 2003), *Brucella abortus* antigen (Zhou *et al*., 2003) and *E. coli* vaccine (Yonash *et al*., 2001). Also, QTL associated with response to specific pathogens have been identified, including Marek's disease virus (MDV) (Yonash *et al*., 1999a; McElroy *et al*., 2005), eimeria (Zhu *et al*., 2003) and salmonella (Mariani *et al*., 2001; Kaiser and Lamont, 2002; Tilquin *et al*., 2005). The results of independent studies often revealed different marker-trait associations. These differences may arise because of biological differences, such as different QTL alleles existing in the original populations or sampling of specific alleles in the formation of resource populations or because of technical issues such as marker spacing and coverage or the size of the tested population limiting power of detection of QTL.

STRATEGIES FOR MARKER-ASSISTED SELECTION

Once molecular markers that are linked to QTL or immune-response genes have been identified, their effects can be estimated based on the association between phenotype and genotype. The resulting estimates can be used to assign a "molecular score" to each selection candidate, which can be used to predict the genetic value of the individual and used for selection. The constitution and method of quantification of the molecular score depends on type of LD that is used, and on how the marker will be used in selection (Dekkers and Hospital, 2001). Methods for incorporating marker data in genetic evaluation procedures are described in Fernando and Totir (2004).

Based on the previously described methods for QTL detection, three types of observable genetic loci for use in QTL detection and MAS can be distinguished, as described by Dekkers (2004):

1. Direct markers: loci that genotype the functional polymorphism for a QTL.
2. LD-markers: loci that are in population-wide LD with a QTL.
3. LE-markers: loci that are in population-wide LE with the functional mutation but in LD on a within-family basis.

The three types of molecular loci differ not only in methods of detection but also in methods of application in genetic selection programmes. Whereas direct and, to a lesser degree, LD-markers, allow selection on genotype across the population, use of LE-markers must allow for different linkage phases between markers and QTL from family to family. In addition to a molecular score, individuals can also obtain a regular estimate of the breeding value for the collective effect of all the other genes (polygenes) on the trait. Thus, in general, at the time of selection, both molecular and phenotypic information is available for use in selection. The following three selection strategies can then be distinguished:

1. Select on the molecular score alone.
2. Tandem selection, with selection on molecular score, followed by selection on phenotype-based estimates of breeding value.
3. Selection on an index of the molecular score and the regular phenotype-based evaluation.

Selection on molecular score alone ignores information that is available on all the other genes (polygenes) that affect the trait and is expected to result in the lowest response to selection, unless all genes that affect the trait are included in the molecular score. This strategy, however, does not require additional phenotypes, other than those that are needed to estimate marker effects, and can be attractive when phenotype is difficult or expensive to record, as is the case for disease and survival traits.

If both phenotypic and molecular information is available on selection candidates, index selection is expected to result in greater response to selection than tandem selection. The reason is similar to why two-trait selection using independent culling levels is expected to give lower multiple-trait response than index selection; two-stage selection does not select individuals for which a low molecular score may be compensated by a high phenotype-based evaluation. The optimum choice between these selection strategies (and other alternatives) also depends on other factors, such as market and cost considerations. It is often difficult to estimate the economic values for enhanced genetic resistance to disease, because the extent of exposure of production populations to pathogens may be unpredictable, and even asymptomatic levels of pathogens can reduce efficiency of production. Additionally, it is difficult to assign economic value to the ethical aspects of the improved animal health and well-being that result from genetic enhancement of resistance to disease.

FUNCTIONAL GENOMICS

One of the main goals of post-genome research is to identify the function of the different genes encoded by the genome of organisms. This includes not only the predicted 20–25 000 protein coding genes, but also the very important non-coding regulatory RNA molecules. While this is clearly an overwhelming task, technology has kept pace with the information generated during genome projects and promises to facilitate rapid identification of gene function through gene expression on a global scale. This is the era of functional genomics.

Some definitions are required: the *genome* consists of the entire set of genetic material of an organism. The *transcriptome* consists of the mRNA molecules that are transcribed from the genome, and similarly, the *proteome* consists of the set of proteins that have been translated from transcribed mRNA. Both the transcriptome and proteome are of particular interest within a particular physiological, developmental or disease state.

Upon completion of the chicken genome sequence, the transcriptome was characterized and cDNA- and EST-based evidence presented for almost 12 000 chicken protein coding genes and 6300 non-coding mRNA (Hubbard *et al*., 2005). Further analysis of these data has provided a wealth of information on genes of the chicken immune system, revealing the identity of chicken immune-related genes ("the immunome"), including those that are shared with mammals and those that are lacking or are unique to birds (Smith *et al*., 2004; Kaiser *et al*., 2005). With these resources, we are now equipped to address research questions in avian immunology on a whole genome scale.

DNA microarrays were designed to provide quantitative information on the levels of mRNAs and have become a powerful tool for analysing the global changes in gene expression that underlie complex biological responses. A DNA microarray consists of cDNA or oligonucleotides (targets) complementary to known genes immobilized on a solid support, usually nylon, glass or silica. Fluorescent, chemiluminescent or radioactively labeled material derived from mRNA is hybridized to the immobilized targets, and the intensity of the signal is a relative estimate of gene expression. In theory the expression profile of an organism's complete transcriptome can be evaluated, and by comparing a disease state to a normal state, or the progress of a disease over time, information on genes that play a role in the response can be obtained. Since there is generally (but not always) a tight correlation between the expression patterns of a gene and the protein it encodes, one can extrapolate from gene expression studies to protein function.

Two basic forms of DNA arrays have been developed: spotted arrays in which the cDNA or oligonucleotides are robotically deposited onto the solid surface, or arrays in which the

oligonucleotides are directly synthesized *in situ*. The former type is a very flexible format, allowing researchers to construct their own arrays out of curated clone sets. In addition, the use of cDNA usually allows application to non-homologous, but related species. The latter type has higher cost, but also higher quality control and reproducibility. Affymetrix GeneChips® has been the market leader in this technology. Only very recently, the chicken genome is represented by both types of arrays and these have been used in a limited number of immunological-related studies.

Neiman *et al*. (2001) were among the first to develop a chicken immune system array. This array contained 2200 elements and was used to analyse *myc*-oncogene-induced lymphomagenesis in the chicken bursa of Fabricius. Genes whose expression levels correlated with *myc* expression in transformed follicles and metastatic tumours were identified and the results will guide future studies to look at genes involved in nucleolar function, ribosome biogenesis and protein synthesis. The coverage of this array was subsequently increased to 3451 and used to compare the transcriptional signature of chick bursal lymphomas resulting from avian leukosis virus (ALV) insertional mutation of c-*myb*, and transformation by v-Rel (Neiman *et al*., 2003). Unique and shared pathways were revealed, as well as different profiles for short and long latency lymphomas. These arrays were also used to identify genes regulated by the v-*jun* oncogene in chicken embryo fibroblasts (Black *et al*., 2004) and generate a pattern of expression that is strikingly similar to one produced by the MDV oncogene, *meq* (Levy *et al*., 2005). Collectively, these studies establish common mechanisms for transformation of chicken cells, and point to differences that are characteristic of individual pathogens, as well.

Another immune system array has been used to study the host response to infection with MDV (Morgan *et al*., 2001) and herpesvirus of turkeys (HVT) (Karaca *et al*., 2004) and to catalogue gene expression in the developing chick thymus (Cui *et al*., 2004). Not unexpectedly, many of the genes identified in both studies are known to respond to interferon. A number of differences were also detected; these could contribute to the pathology of Marek's disease or that vaccinal response of HVT, and are worthy of additional studies.

An avian macrophage-specific array containing almost 5000 genes expressed in macrophages has been used to examine the transcriptional response of macrophages to Gram-negative bacteria in comparison to the response to lipopolysaccharide (Bliss *et al*., 2005). Bacteria elicit a more complex response; there is common signalling through TLR-4, but additional pathways are activated by whole bacteria. The same array has been used to evaluate the responses of avian macrophages to several species of *Eimeria* (Dalloul *et al*., 2007). Another avian immune system array has been used to evaluate host response to different respiratory pathogens (Munir and Kapur, 2003; Dar *et al*., 2005). A microarray analysis of chicken intestinal lymphocyte genes induced or repressed in response to infection with eimeria has also been reported (Min *et al*., 2003).

Microarrays have the potential to be used for identification of candidate genes for desired traits. Liu *et al*. (2001) have used microarrays to identify differentially expressed genes in MDV-resistant lines of birds and successfully integrated the microarray results with genetic mapping data to identify candidate genes for resistance.

In another application of this technology, Degen *et al*. (2003) have used microarrays for identification of host-derived natural adjuvants. Enhancement of the immune response is a major issue in vaccine development and the use of natural adjuvants is more desirable than commonly used chemical adjuvants. Moreover, global profiling can be used not only to identify candidates, but also to evaluate their effectiveness.

The elements on two of the early immune system arrays were consolidated and combined with genes from a wide range of tissues to construct a 13 000 element array (Burnside *et al*., 2005), which has been widely distributed to the avian research community. In addition, Affymetrix has recently developed a chicken GeneChip® with coverage of 28 000 chicken genes and avian pathogen genes. Now that these tools for global gene expression assessment are readily available, we can expect a wave of microarray studies in avian immunology in the near future.

Microarrays are not the endpoint of an experimental design, but are a conduit to developing testable hypotheses. It is important to appreciate the inherent strength of microarray

analysis in that they generate unbiased results and frequently provide unexpected insights. Therefore, this approach is not a substitute for hypothesis-driven biological investigation, but is destined to become a prerequisite for future research. Genes identified by microarray analysis as being important in the experimental paradigm under study should be further characterized in a variety of ways, including complementing gene expression with protein expression, and ultimately, their roles can be evaluated or verified in transgenic animals.

TRANSGENIC ANIMALS

A transgenic animal is one in which there has been a deliberate modification of its genome. A foreign gene, termed the "transgene" is introduced in such a way that it will be transmitted through the germline and will either be expressed in every cell in the mature animal or in selected tissues or at selected times in development.

The development of agriculturally relevant transgenic animals has the potential to accelerate conventional breeding programmes for improvements in desirable production traits. Relevant to avian immunology is the possibility of developing disease-resistant birds. In theory, this could be accomplished in a myriad of ways – e.g. (1) by enhancing expression of genes that are fundamental for the innate immune response, birds might display resistance to pathogens or (2) by inactivating viral receptors, birds could be resistant to viral pathogens. However, because many viral receptors have other physiological functions in the host, transgenic animals with alterations in viral receptor activity should be carefully evaluated for any unintended consequences.

Technology for the development of transgenic chickens has lagged behind that of mammals, in large part due to the differences in reproductive biology. In mammals the single cell oocyte is the starting place for introduction of the transgene, and this ensures that after cell division and embryo formation takes place, all subsequent cells will harbour the introduced gene. Because of the intricate processes involved in egg laying, a single cell chicken oocyte is not available for manipulation. An egg is certainly readily accessible, but the embryo represents $>50\,000$ cells at this stage. This vast difference has hampered progress until very recently, with the development of viral vectors for the efficient introduction of transgenes and establishment of transfectable chicken embryonic stem-cell (ESC) lines.

An excellent and recent review of transgenic chickens research covers developments through 2003 (Mozdziak and Petitte, 2004) and will not be repeated here. Since then, considerable progress has been made using either retroviruses or transfected ESCs. The improved efficiencies and the ability to generate tissue-specific expression promise to make transgenic birds a more widely used technique.

Expression of virally introduced transgenes has been improved through the use of lentiviral vectors. Lentiviruses are a complex retrovirus subfamily that have the ability to incorporate foreign DNA into both dividing and non-dividing cells (Naldini *et al.*, 1996) and produce high titres of viral particles. Lentiviral vectors can accommodate up to 9000 nucleotides of foreign genes and produce stable and sustained gene expression. Based on high success rates in production of transgenic mice, lentiviral vectors have been used successfully to create transgenic chickens (McGrew *et al.*, 2004). An equine infectious anaemia lentivirus vector containing the green fluorescent protein (GFP) reporter gene was used to infect embryos and an efficiency in the order of 100-fold higher than any previously published method was obtained. Importantly, the GFP transgene showed expression in both the G1 and G2 generations.

The lentivirus system has been refined for tissue-specific expression. Scott and Lois (2005) used human immunodeficiency virus-derived lentiviruses to produce transgenic quail expressing GFP under control of the human synapsin gene I promoter. The transgenic birds express the reporter gene in neurones and have respectable germline transmission rates. The approach should be readily transferable to chickens and is an important advance in that it will allow tissue-specific expression of desired transgenes, or directed knockout of deleterious genes.

Another approach to the development of transgenic chickens is through the use of ESC. ESC are undifferentiated, pluripotent cells derived from very early, blastocyst-stage embryos. These cells have no predetermined lineage and have the potential to develop into any somatic or germ cell. The key advantage of ESC cells is that they can proliferate in cell culture, and can integrate foreign DNA at specific, preselected sites, and cells harbouring the introduced DNA can be selected. The ESCs are then injected into oocytes, where they retain totipotency and can develop into all tissues, including germ cells. The transgene is then transmitted to subsequent generations.

A very significant advance in the application of ESC technology to birds focused on the development of chicken ESC lines (van de Lavoir *et al.*, 2006b), which were subsequently applied to develop the chicken as a bioreactor for the production of therapeutic monoclonal antibodies (mAb) (Zhu *et al.*, 2005). The ESC lines can be cultured and transfected with plasmid DNA. Introduction of the transfected cells to the subembryonic cavity and culture of the embryo in surrogate shells produced birds with transgene expression in somatic tissues. In a practical application of this approach, the genes encoding a human mAb and the regulatory sequences restricting its synthesis to egg white were transfected into ESCs, which were cultured and selected for the transgene, and ultimately introduced into chick embryos. This produced chimaeric birds that lay eggs containing milligram amounts of antibody, which can be separated from the egg white proteins to produce a purified product useful as a human pharmaceutical. Germline transmission of chicken primordial germ cells that have been genetically modified has recently been achieved (van de Lavoir *et al.*, 2006a). This technology opens up new frontiers for poultry science with the potential of using chickens for "pharming".

These recent successes in producing transgenic birds now mean that this technology is likely to become a routine effective way to modify the genome of birds. Microarray studies can lead to the identification of potentially important target transgenes.

POTENTIAL USES AND IMPACT OF TRANSCRIPTOMICS AND PROTEOMICS

Although in its infancy, the application of high throughput technologies to avian immunology has already provided novel data and generated important hypotheses. In the future, we expect that functional genomics will deepen our understanding of the avian immune system and provide the functional information that is needed to improve annotation and understanding of the role of immune function genes in biology. A comprehensive gene expression profile can provide a detailed picture of the complex mechanisms underlying host–pathogen interactions, the immunological process and disease and the relationship between genetic variation and disease resistance. In addition, microarray analysis can be used to characterize differences in strains of pathogens exhibiting higher virulence. The combination of microarrays with analysis of genome-wide molecular marker genotyping (genetical genomics) will facilitate the mapping of expression-QTL (eQTL), the loci that control the expression of genes (de Koning *et al.*, 2005).

One of the key strengths of DNA microarray technology is the ability to follow gene expression patterns over time. Studies with respect to time are critical in immunology, since the immune system implements its response in stages: first it responds to infectious agents by recognizing antigens that are unique to the pathogen (innate immunity), but are absent from self (tolerance); then a secondary response gives rise to immunological memory (adaptive immunity). As more microarray studies emerge we can expect to see a common group of genes that comprise a stereotypic response to pathogens, e.g. the inflammatory response. Different pathogens have probably evolved different mechanisms to evade host defenses, and these can be revealed by the molecular signature of gene expression. Novel pathways that play a role in disease pathogenesis are apt to be identified and this will establish the framework for designing new therapeutic strategies.

It is important to keep in mind that mRNA levels do not always correlate with protein levels and it will be necessary to link microarray data to protein data to obtain a clear picture

of cell activity. High throughput, automated protein analysis, using a combination of two dimensional gels and mass spectrometry is rapidly becoming a widely available technology. Both technologies present challenges in reproducibility, data management and cost, but these challenges can be dealt with. The potential of functional genomics and proteomics is, quite simply, enormous.

The combination of discoveries from transcriptomics and proteomics, with assessment of structural variation in the genome and the associations with immunological traits, is expected to enhance our fundamental understanding of the genetics of avian immunology. This research will also provide tools to enhance avian health by genetic selection of breeding stock, individual gene modification and improved vaccines and immunomodulators.

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12 THE AVIAN MUCOSAL IMMUNE SYSTEM

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With very few exceptions (e.g. pathogens transmitted via the germline) animal pathogens enter the host by breaching the barrier between the external and internal milieus. This barrier consists of specialized tissues characterized by two main components: an externally located epithelium and the underlying connective tissue. The nomenclature for the epithelium and connective tissues depends on the particular surface covered by the barrier. For instance, the epithelial tissue covering the external body surface is referred to as the epidermis and its underlying tissue the dermis; both together form the skin. Some of the immune protection mechanisms associated with the skin have been described in Chapters 7 and 9. The surface layer involved with the digestive respiratory and reproductive tracts is referred to as epithelium and the underlying tissue the lamina propria. The combination of these two tissues forms the mucosa, mucosal membrane or mucosal surface.

The combined mucosal surfaces of the gut, respiratory and reproductive tracts represent by far the largest surface area in contact with the external milieu. These can also be considered as the largest organ system in vertebrates, each of these tracts being involved with diverse physiological functions. The protective mechanisms vary widely, from barrier functions (e.g. skin and ciliated cells in the trachea) to highly specialized dendritic (Langerhans) cells in the skin epithelium and specialized lymphoid tissues (see Chapter 2). To protect the body from infection within the mucosal immune systems of the gut, respiratory and reproductive tracts have highly developed lymphoid tissues such as the gut-associated lymphoid tissue (GALT) and bronchial-associated lymphoid tissues (BALT). In addition, there are well-developed immunological activities that provide essential protection in the different parts of these systems. Within the gut, especially, there are different immunological requirements in different locations, because of the nature of the different local conditions and the specialized functions within different regions. In mammalian species, the GALT contain more lymphocytes than secondary lymphoid tissues, such as the spleen and lymph nodes. It is likely that this is also the case in avian species (reviewed by Schat and Myers, 1991). In addition to the protection against pathogens, these mucosal tissues have other specialized primary functions such as digestion, respiration and reproduction. The mucosal surfaces have a number of common features. Since each forms a major barrier between the external environment and internal milieu, they provide an important portal of entry for pathogens. This is especially the case with the gut and respiratory tract where the continuous movement of external substances – nutrients and air, respectively – and the need to transport or exchange essential molecules across the mucosal surface for organs to function properly and the animal remain healthy. Hence, there is a continual challenge from new materials/organisms which pass through the system. The problem is further exaggerated within the gut where, apart from essential substances some organisms (mainly bacteria) may reside and have a beneficial effect on digestive processes, while pathogenic organisms can replicate in

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the mucosal epithelial cells or cross the mucosal surface to enter the body proper and cause disease. Although the mucosal immune systems have many features in common, the very special features of the digestive, respiratory and reproductive tracts are far too diverse to be dealt with together within a single chapter. Each one is therefore described separately in the following substantive chapters.

REFERENCE

Schat, K.A. and Myers, T.J. (1991). Avian intestinal immunity. *CRC Crit. Rev. Poult. Biol.* 3, 19–34.

13

THE AVIAN ENTERIC IMMUNE SYSTEM IN HEALTH AND DISEASE

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GENERAL CONSIDERATIONS
GUT STRUCTURE AND IMMUNE COMPARTMENTS
DEVELOPMENT OF THE ENTERIC IMMUNE SYSTEM
DEVELOPMENT OF IMMUNE RESPONSES TO MODEL ANTIGENS
IMMUNITY TO ENTERIC PATHOGENS
MATERNAL ANTIBODY AND PROTECTION OF THE YOUNG CHICK
VIRAL INFECTIONS OF THE GUT
BACTERIAL INFECTIONS OF THE GUT
PARASITIC INFECTIONS OF THE GUT
CONCLUDING REMARKS
REFERENCES

GENERAL CONSIDERATIONS

The intestine is a structurally complex organ that has a primary role in the acquisition of nutrients and water, with physiologically and structurally distinct regions specialized in different digestive and/or absorptive functions (e.g. the stomach, small or large intestinal regions). The digestive/absorptive functions of different parts of the intestine are outwith the remit of this chapter but those interested in gut immune function will find it useful to become familiar with these aspects of intestinal physiology. The gut is a major site of development, residence and portal of entry for pathogenic microorganisms into the deeper body tissues, therefore any perturbation of gut physiology often results in substantial clinical consequences. Hence, an effective immune capability in the gut is essential to combat the plethora of pathogenic microorganisms that reside in, or pass through, this tissue.

Various aspects of gut physiology are important for limiting establishment of pathogens, including the acidity of the stomach and the activity of digestive enzymes or bile constituents. However, many microorganisms also use gut physiological features as triggers for development and maturation. For example, the excystation or hatching of protozoa and helminths from the transmission stages (oocysts, cysts or eggs) is triggered by conditions such as temperature, short exposure to acidic environments, the physical disruptive capacity of the gizzard and/or exposure to enzymes and bile components. Similarly, these environmental changes trigger physiological events in many bacterial species that facilitate their survival and colonization of the intestine.

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Although the positive effects of the immune system in combating pathogens are often a focus for the infectious disease immunologist, the aetiology of enteric disease is often linked to inappropriate immune activity. Indeed, with many infections it is difficult to separate the physical damage associated with the direct action of the pathogen from the damage associated with the immune response generated against the pathogen. The intestine is highly sensitive to damage mediated by ongoing immune responses and in many cases the immune response is the major cause of intestinal pathogenesis in mammals and birds (e.g. Strober *et al.*, 2002). Classical signs of an ongoing immune response in the gut include increased leukocyte infiltration into the lamina propria and changes in gut structure such as villus atrophy and hyperplasia of enterocytes in the crypts of Lieberkühn.

One of the most important gut-specific features affecting immune function is the requirement not to respond inappropriately to food derivatives or the vast biomass of non-pathogenic microorganisms that inhabit the gut. Indeed, the aetiology of inflammatory bowel diseases in humans is a dysregulation of gut immune responses, especially those driven by the constituents of commensal organisms or food. Mechanistically, there are at least two groups of effects that contribute to the control of potentially harmful responses against non-pathogen-derived antigens in the gut; the first being a failure to respond due to lack of exposure of a particular antigen (oral ignorance) and the second due to some form of active prevention or modulation of a response (oral tolerance). The requirement not to respond to the majority of foreign material presented in the gut is a feature that marks this immune environment as unique.

In the following sections we will deal with the structure and function of the gut immune system of birds with particular reference to development and function of the immune response against major groups of enteric pathogens (including viruses, bacteria and parasites). Unfortunately, with many avian pathogens the immune responses induced by infection, and those that operate to kill the invading pathogen are not well defined and much of our fundamental knowledge has been gained from studies on a small group of selected pathogens which infect the chicken. The work on avian enteric pathogens has been dominated by studies on the enteric bacteria (principally *Salmonella enterica*) and with the protozoan parasites of the genus *Eimeria*, which cause enteric coccidiosis. However, the increased prominence of avian influenza (AI), which infects both the gut and respiratory mucosa, as well as systemic sites in the case of high pathogenicity strains (see Chapter 14), should stimulate significant investigations into the immunobiology of enteric viral infections.

GUT STRUCTURE AND IMMUNE COMPARTMENTS

The structure and organization of intestinal immune tissue varies along the length of the gut and most studies have focussed on structures associated with the small and large intestine. The basic organization of immune compartments in the gut is given in Figure 13.1. Essentially, the gut is a tubular structure enclosed by a single layer of polarized epithelial cells affixed to an extracellular matrix known as the basement membrane. The epithelial cells are held in tight juxtaposition by complex intercellular structures, collectively known as "tight junctions" (see Hermiston and Gordon, 1995a for a detailed review of these interactions). To increase the functional surface area, many parts of the gut are convoluted to form protruding villus structures interspersed by indentations known as crypts. The villus/crypt unit is highly zonal in organization and enterocytes of many different types, or stages of differentiation, are found at distinct locations within the villus or crypt. The proliferative zone lies within the crypt unit and the epithelial stem cells divide to form daughter cells that differentiate during migration along the villus structure or deeper into the base of the crypt. In an elegant set of studies using the mouse, it was shown that enterocytes derived from each crypt are responsible for feeding enterocytes into four villi, and each villus was formed from cells derived from four crypts (Hermiston and Gordon, 1995b). The enterocytes derived from the crypts migrate along the villus structure in a spiral path and survive for between 48 and 96 h (dependent on the length of the villus and age

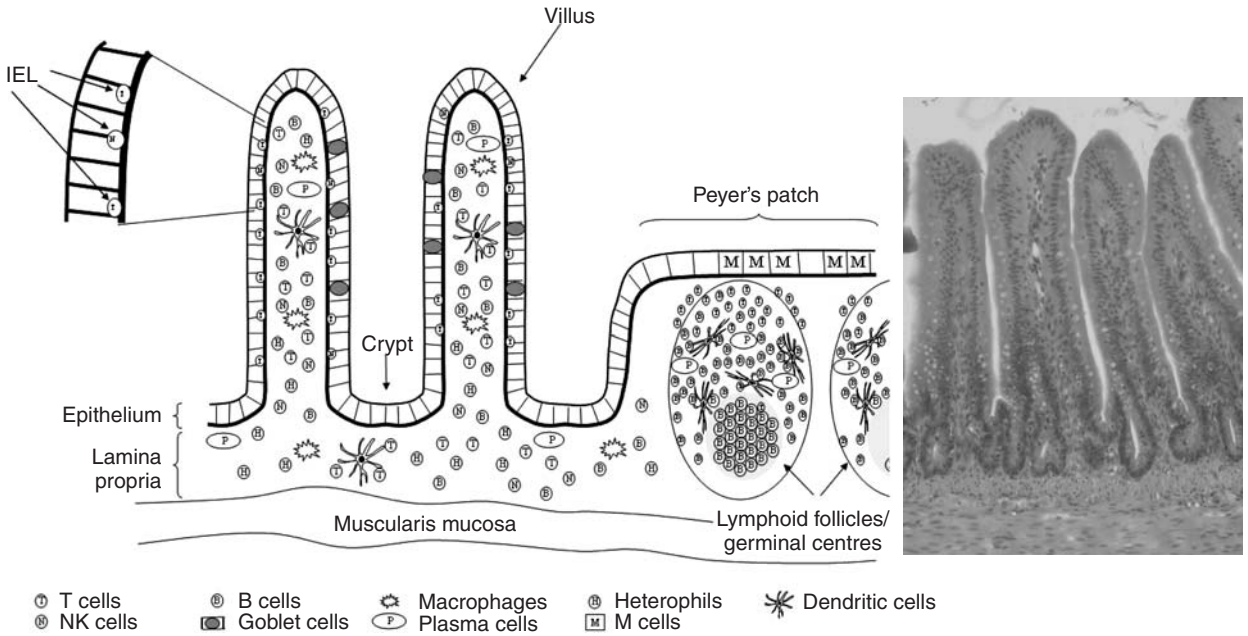

FIGURE 13.1 (a) Schematic representation of the organization of immune cells in the small intestine and (b) photo-micrograph of a chicken ileum depicting intestinal villi and crypts (×100 magnification).

of the chicken) before being lost by apoptosis from the villus tip (Cook and Bird, 1973; Smith and Peacock, 1989; Uni *et al.*, 1995, 2000).

Reactions against pathogens and the intestinal tract may be initiated by interactions occurring at the most proximal sites associated with the mouth and buccal cavity, and the small lymphoid aggregates in these tissues (Ohshima and Hiramatsu, 2000; Olah *et al.*, 2003) which may be important to prime responses at more distal sites, as well as performing a local protective function. The proventriculus (glandular stomach) and gizzard are sparsely populated with immune cells and little information is available documenting the ability to mediate immune responses in these tissues. In contrast, numerous studies have examined the function of the immune system in the small intestine (duodenum, jejunum and ileum), and large intestine including the caeca.

Three-dimensional villus/crypt or equivalent structures in many regions of the intestine pose an additional constraint on the function of the immune system, increasing the physical area which needs to be protected and acting as barriers to lateral movement of cells. The gut-associated lymphoid tissues (GALT) form an organized series of structures that comprise part of the more extensive mucosa-associated lymphoid tissues (MALT; described in Chapter 2) which also includes nasal, lung and reproductive tract tissues (see Chapter 14). The homing of immune cells is governed by interactions between molecular addressins on tissue vessel walls (e.g. high endothelial venules) with immune cell-expressed integrins and adhesins as well as the ability of the immune cells to respond to particular chemokines and other chemoattractant molecules (described in Chapter 10). The nature of these interactions links different areas of the mucosal immune system, and cells stimulated in one mucosal site migrate through other mucosal sites. Although many of the important molecules have not been identified in birds the recent completion of the *Gallus gallus* genome sequence (www.ensembl.org/Gallus_gallus/index.html; International Chicken Genome Sequencing Consortium, 2004) offers the prospect of rapidly identifying those molecules and processes most relevant to the chicken.

Chicken GALT Structures

The GALT comprises lymphoid cells residing in the epithelial lining and distributed in the underlying lamina propria as well as specialized lymphoid structures located at strategic sites

along the gut. The definable structures include lymphoid aggregates located within the lamina propria, Meckel's diverticulum, Peyer's patches (PP) and caecal tonsils (CT) (see Plates 2.1–2.2).

The GALT is a key immunological system estimated to comprise more immune cells than any other tissue (Kasahara *et al.*, 1993b) with the associated structures forming a site to promote co-localization of the many immune cell types required to initiate and mediate immune function. There is also considerable cellular traffic between different gut immune structures and the systemic sites including the bone marrow and spleen. Many of the organized GALT structures are sites of immune induction (Jeurissen *et al.*, 1994; Bar-Shira *et al.*, 2003; Bar-Shira and Friedman, 2005) providing conditions necessary to induce appropriate immune responses (e.g. immunoglobulin (Ig)A production by B cells).

Chickens lack highly structured lymph nodes, like those found in mammals, but have a number of distinct lymphoid aggregates that line the length of the gut (see Chapter 2). In their simplest form these consist of a specialized epithelium containing microfold cells (M cells) which sample the gut lumen contents and deliver them to underlying macrophages and dendritic cells (Burns and Maxwell, 1986; Gallego *et al.*, 1995; Jeurissen *et al.*, 1999; Kitagawa *et al.*, 2000). In close proximity to these cells, and with varying degrees of organization, are follicles rich in B and T lymphocytes. The chicken foregut is considered to have relatively little organized lymphoid tissue compared with the hindgut (Befus *et al.*, 1980) although oesophageal tonsils have been described, located at the junction of the oesophagus and the proventriculus (Olah *et al.*, 2003). Up to eight tonsillar units have been described; each consisting of a crypt surrounded by dense lymphoid tissue organized into B and T cell regions. Organized lymphoid tissue is also located throughout the entire lamina propria of the proventriculus above the glandular units (Jeurissen *et al.*, 1994).

The unique avian lymphoid structure, Meckel's diverticulum, develops at the junction of the duodenum and jejunum in the young chick (Olah and Glick, 1984). Although the function of this lymphoid tissue is not completely understood, since it is formed from the lining of the vitelline duct, it has been suggested that it is important for preventing pathogen transmission from the yolk sac in the newly hatched chick (discussed in Chapter 2). Several lymphoid structures with the characteristics of mammalian PP can be found scattered throughout the intestine (Befus *et al.*, 1980) comprising a dome of specialized epithelium (M cells) overlaying structured follicles with clearly defined T and B cell areas. Although not evident at hatching, chicken PP become visible to the naked eye in the second week (Befus *et al.*, 1980; Burns, 1982) and increase in number with age. The PP anterior to the ileo-caecal junction is the most prominent and is enlarged after many gut infections. Within the large intestinal region, CT are located in the neck region of each of the caeca and represent the most studied structures of the avian GALT. The CT are large lymphoid aggregates, structurally similar to the PP, that contain multiple follicles and are overlaid by M cell-rich epithelium (Kitagawa *et al.*, 1998, 2000). Structurally less well defined, isolated lymphoid follicles are present throughout the small and large intestine with greatest density in the apical region of the caecum (Del Cacho *et al.*, 1993; Kitagawa *et al.*, 1996, 1998). The colon is less well endowed with lymphoid structures but follicles have been observed in the proctodeal and urodeal regions of the cloaca (Befus *et al.*, 1980) and are particularly abundant around the bursal duct (Friedman *et al.*, 2003).

The bursa of Fabricius, a lymphoepithelial outgrowth from the proctodeum, is one of the primary lymphoid organs of birds primarily responsible for B lymphocyte development (reviewed in Ratcliffe, 2006). However, the antigen sampling capacity of the bursal tissue is well established (Schaffner *et al.*, 1974; Sorvari *et al.*, 1975) and this, in addition to the presence of T cells, has led to the suggestion that the bursa could also have a role as a secondary lymphoid organ (Friedman *et al.*, 2003; Bromberger and Friedman, 2005). The process of retrograde peristalsis, usually considered as a means of increasing water conservation from kidney secretions entering the cloaca, could also be the means by which bursal follicles sample hindgut bacteria (Friedman *et al.*, 2003) and cloacally applied antigens enter the bursa and induce specific B cell responses in the peripheral tissues (Ekino *et al.*, 1979), this has been described in Chapter 2. The influence of gut-derived antigens or products of the microflora that act as B cell mitogens in normal post-hatch B cell development is considered elsewhere (Ratcliffe, 2006; chapter 4).

Cellular Composition of the Avian GALT

The avian GALT comprises a diverse set of cell subsets, distinct from that seen in systemic tissues, but includes representatives of each of the major cell populations found in other sites. Overall the gut is populated with heterophils, macrophages, dendritic cells, natural killer (NK) cells, and B and T lymphocytes, although the proportions of each cell type differ widely according to locality and age. Other factors that affect the composition and surface phenotype of the gut-associated immune cell populations include the natural intestinal microbiota, diet, host genetics and exposure to pathogenic microorganisms. In addition to the cell populations found in distinct gut structures the epithelial layers of the gut are populated with a highly specialized group of lymphocytes collectively known as the intraepithelial lymphocytes (IEL).

The composition of small intestinal IEL population in a healthy adult bird includes major subsets of NK cells and T cells bearing the $\gamma\delta$ or $\alpha\beta$ form of the T cell receptor (TCR) (Lillehoj, 1994; Göbel *et al.*, 2001). In contrast to other tissues, B cells are almost entirely absent from the IEL and the T cells predominantly express the CD8 co-receptor with smaller populations of TCR$\alpha\beta^+$ CD4$^+$ and CD4$^+$CD8$^+$ cells (Vervelde and Jeurissen, 1993; Lillehoj *et al.*, 2004). Moreover, within the CD8$^+$ IEL population the majority express CD8$\alpha\alpha$ homodimers, rather than the CD8$\alpha\beta$ heterodimer commonly expressed on classical CD8$^+$ T cells found at systemic sites (Vervelde and Jeurissen, 1993; Imhof *et al.*, 2000; Lillehoj *et al.*, 2004). Very few heterophils are present in the epithelial compartment of healthy chickens but infection can lead to translocation of these cells into, and across, the epithelial barrier (Henderson *et al.*, 1999; Kogut, 2002; Van Immerseel *et al.*, 2002; Bar-Shira and Friedman, 2005).

The lamina propria underlies the epithelial layer and is highly populated with a wide range of different leukocytes including granulocytes, macrophages, dendritic cells and lymphocytes of B and T cell lineages. Numerically, B and T cells are the most common lymphocytes (~90%); the remainder being the NK cell phenotype. In contrast to the IEL population, the T cell population of the lamina propria contains a smaller proportion of $\gamma\delta$ T cells (~10%) with the much larger $\alpha\beta$ T cell population dominated by CD4$^+$ T cells, with a less prominent CD8$^+$ cell population. Many of the B cells in the lamina propria have undergone class switching to the secretory IgA isotype; IgA is present at high concentrations in intestinal fluids and bile (3.5–12 mg/ml) compared with IgM (<100 μg/ml; Lebacq-Verheyden *et al.*, 1972; Bienenstock *et al.*, 1973b; Mockett, 1986). In serum, IgA is predominantly in monomeric form whereas in secretions it is found in a polymeric configuration (Bienenstock *et al.*, 1973b). Directional transport of IgA across epithelia is mediated by secretory component interactions with the polymeric Ig receptor (Bienenstock *et al.*, 1973a; Parry and Porter, 1978; Rose *et al.*, 1981; Peppard *et al.*, 1986). The cellular composition of the PP and the CT is essentially similar to that found in the lamina propria.

Chickens have two families of TCR$\alpha\beta^+$ cells expressing either Vβ1 (identified by the anti-TCR2 monoclonal antibody) referred to as the TCR$\alpha\beta_1$ subfamily and those expressing Vβ2 (identified by anti-TCR3 monoclonal antibody) referred to as the TCR$\alpha\beta_2$ subfamily. Interestingly, TCR2$^+$ cells predominate within the IEL compartment and are relatively abundant in the intestinal lamina propria (in contrast both TCR2$^+$ and TCR3$^+$ populations are abundant in the spleen). Depletion of TCR2$^+$ T cells led to a deficiency in IgA production in the gut but did not affect systemic IgM or IgY responses suggesting a differential role for TCR$\alpha\beta^+$ T cells according to TCRVβ usage (Chen *et al.*, 1989; Cihak *et al.*, 1991). As in mammals, higher numbers of TCR$\gamma\delta^+$ (identified by the anti-TCR1 antibody) T cells are found within the IEL, although birds also express much higher numbers of TCR$\gamma\delta^+$ T cells in the periphery compared with mouse or man (Cooper *et al.*, 1991). In the periphery, most TCR$\gamma\delta^+$ T cells do not express either CD4 or CD8, whereas those in the gut are predominantly CD8$^+$ (Sanchez-Garcia and McCormack, 1996). Whilst the biological functions of avian TCR$\gamma\delta^+$ T cells are still unclear, *in vitro* studies have demonstrated that they are capable of cytotoxicity (Chen *et al.*, 1994) and may also have immunoregulatory functions (Quere *et al.*, 1990). Purified chicken TCR$\gamma\delta^+$ T cells respond poorly to stimulation with mitogens or TCR cross-linking (Sowder *et al.*, 1988; Cooper *et al.*, 1991). TCR$\gamma\delta^+$ T cell proliferation can be achieved by co-culture with TCR$\alpha\beta^+$ T cells (Arstila

et al., 1993), or by providing an exogenous source of interleukin-2 (IL-2) or other growth factors (Kasahara *et al.*, 1993a).

The Enterocyte as Part of an Integrated Gut Immune System

The enterocytes are often overlooked when considering the function of the gut immune system. It is clear that these multi-functional epithelial cells play an important role in the organization and function of the enteric immune system. Studies using the mouse model have shown the importance of enterocyte-expressed chemokines in the recruitment and retention of IEL cell populations (Onai *et al.*, 2002; Hosoe *et al.*, 2004). In a more direct manner, enterocytes form the physical barrier between the host and the gut lumen and interact directly with the gut microbial flora in the gut by mucus production and secretion of antimicrobial molecules (e.g. defensins). In mammals, the specialized enterocytes in the crypts are recognizable by prominent cytoplasmic granules called Paneth cells (Ganz, 2000). Paneth cells secrete lysozyme, phospholipase A_2 as well as the α- and β-defensins, which all have antimicrobial activity *in vitro* against bacteria (Selsted *et al.*, 1984), fungi (Selsted *et al.*, 1985; Lehrer *et al.*, 1988) and some enveloped viruses (Lehrer *et al.*, 1985; Daher *et al.*, 1986). In ostriches, Paneth cells were found to be absent (Bezuidenhout and Van Aswegen, 1990) using classical staining methods, and cells are sparse or not present in chickens. The chicken defensin repertoire is restricted to the β-defensins (Zhao *et al.*, 2001; Higgs *et al.*, 2005). To date, expression of defensins has been reported with heterophils and macrophages (Evans *et al.*, 1994; Zhao *et al.*, 2001; Sugiarto and Yu, 2004) but not epithelial cells. Hence, innate enterocyte-mediated protection of the gut of chickens may be structured differently to that of mammals; this subject deserves attention.

Enterocytes can influence the induction of enteric immune responses by virtue of their expression of pattern recognition receptors (PRR) including the Toll-like receptors (TLR; see Chapter 7). Discrimination between molecular patterns (agonists) associated with pathogenic microorganisms and those found on "commensal" microorganisms is, at least in part, mediated by the polarization of PRR or their ability to respond to agonists (reviewed in Harris *et al.*, 2006). Specialized epithelium composed of M cells overlies many of the lymphoid follicles, PP and the CT and has a primary function of sampling the gut lumen and delivering antigens to classical antigen presenting cells (see Chapter 9). All enterocytes express major histocompatibility complex (MHC) class I molecules and can be induced to express MHC class II molecules (especially under inflammatory conditions), hence these cells can interact with both $CD8^+$ and $CD4^+$ $TCR\alpha\beta^+$ T cells. Similarly, direct interactions between epithelial cells and $TCR\gamma\delta^+$ T cells may be mediated by non-classical MHC molecules, or by expression of a variety of stress molecules at times of epithelial damage. Unfortunately there is little evidence of these interactions in the chicken but studies in the mouse indicate a potential role in regulating epithelial homeostasis (Boismenu and Havran, 1994) and the response to enteric challenge (Roberts *et al.*, 1996; Ramsburg *et al.*, 2003).

DEVELOPMENT OF THE ENTERIC IMMUNE SYSTEM

During the first 5 days after hatching extensive enterocyte proliferation leads to the formation of the basic structure of the gut (e.g. the crypt-villus unit in the small intestine; Uni *et al.*, 2000; Geyra *et al.*, 2001). Enterocytes also mature during this period, increasing in size and adopting a columnar phenotype with defined microvillus structures on the luminal face by 24 h post-hatching (Geyra *et al.*, 2001; Friedman *et al.*, 2003). Differentiation of enterocytes into the mucus-producing goblet cells occurs prior to hatching but their numbers increase rapidly in the post-hatching period (Uni *et al.*, 2003). Moreover, the mucin composition of goblet cells also changes during the early post-hatch period with increasing amounts of neutral mucins (acidic mucins predominate pre-hatch). The stimulus for these changes is unknown but may involve the colonization of the gut with microbial flora and/or components of food. Indeed, when food

was withheld for 48 h higher levels of intracellular mucin were detected in the chick gut, which could either be attributed to reduced mucin secretion or enhanced mucin production (Uni *et al.*, 2003). Structurally, the chicken gut continues to develop for at least 3–4 weeks after hatching with increased complexity of intestinal epithelium (e.g. villus length), the lamina propria, PP and CT also become more defined (Lilburn *et al.*, 1986; Vervelde and Jeurissen, 1993; Uni *et al.*, 1995; Friedman *et al.*, 2003).

Several studies have examined the turnover rate of enterocytes in the small intestine in chickens aged 2 days (Imondi and Bird, 1966; Cook and Bird, 1973), 2 weeks (Smith and Peacock, 1989) or 6 weeks (Uni *et al.*, 2000). The time taken for enterocytes to migrate along the length of the villus increases with age (and villus length) from 50 h in 2-day-old chicks (Imondi and Bird, 1966; Cook and Bird, 1973) to greater than 90 h in the jejunum or ileum of 2- or 6-week-old birds (Smith and Peacock, 1989; Uni *et al.*, 2000). One of the most dramatic responses to inflammation in the gut is the increased production of enterocytes in the crypt and the increased loss of enterocytes from the villus tip. This response results in shortened enterocyte life span, crypt hyperplasia and villus atrophy and represents an important mechanism for removal of infected or damaged enterocytes. In murine models of intestinal inflammation the epithelial cell response is dependent on the activity of T cells (Strober *et al.*, 2002). PP and CT are present at hatching but become more prominent and exhibit increased cellular composition with age (Befus *et al.*, 1980; Vervelde and Jeurissen, 1993; Chapter 2). Similarly, the size and cellular complexity of the lamina propria and IEL compartments increases with age (Befus *et al.*, 1980; Vervelde and Jeurissen, 1993; Gomez Del Moral *et al.*, 1998) and is at least in part dependent on the presence of enteric microflora (Honjo *et al.*, 1993; C. Powers, R.K. Beal and A.L. Smith, unpublished observations).

Profound changes in the number and cellular composition of all gut immune cell populations occurs just prior to and shortly after hatching (Befus *et al.*, 1980; Vervelde and Jeurissen, 1993; Gomez Del Moral *et al.*, 1998) partly as a consequence of the export of thymic T cells and bursal B cells. Dunon *et al.* (1997) measured T cell export from the thymus and proposed that the process occurred in a series of three waves, each wave beginning with the export of $TCR1^+$ cells followed 2–3 days later by the export of $TCR\alpha\beta_1^+$ and $TCR\alpha\beta_2^+$ cells (described in Chapter 3). Thus $TCR\gamma\delta^+$ cells are exported to the periphery at 15–17 embryonic incubation days (EID) around the time of hatching and at 6–8 days post-hatching. $TCR2^+$ and $TCR3^+$ cells migrate to the periphery at 18–20 EID, at 2–4 days and at 9–11 days after hatching. The export of B cells from the bursa has been studied by tracking bursal cells using ^{3}H-thymidine demonstrated that B cells begin to be exported from the bursa around 18 EID (Hemmingsson and Linna, 1972; Linna *et al.*, 1972).

At hatching the intestinal tract is populated with few lymphoid cells (Bar-Shira and Friedman, 2005) and within the epithelium the number of lymphocytes increases with age, with peak numbers present by approximately 8 weeks post-hatching (Vervelde and Jeurissen, 1993). In recent studies by Bar-Shira *et al.* (2003), measurements of the expression of specific mRNA produced by different cell subsets has been used to monitor age-related changes in lymphocyte populations in different parts of the gut. Large increases in $CD3^+$ cells were detected at 4 days post-hatch in all parts of the gut. The number of B cells also increased significantly, initially in the small intestine (4 days post-hatching) and then in the large intestine and CT (6 days post-hatching). At hatching and shortly afterwards the level of B cell-specific mRNA expression was greatest in the CT. These authors also suggested that GALT lymphocytes are functionally immature at hatching, as indicated by the levels of mRNA for IL-2 and interferon-γ (IFNγ). Functional maturity of both B and T cells appears to occur in a biphasic manner, with the first stage occurring during the first week after hatching and the second stage during the second week (Bar-Shira *et al.*, 2003). Many of these changes seem to be a consequence of the colon-ization of the gut with microbial flora, for germ-free chickens have a less well-defined GALT (Honjo *et al.*, 1993; C. Powers, R.K. Beal and A.L. Smith, unpublished data).

During the first 4 weeks after hatching there are also important changes in the proportions of different lymphocyte subsets within the different gut compartments. For example, there is

a shift towards increased numbers of TCR$\gamma\delta^+$ cells in the IEL compartment and also an increase in expression of CD8α (Lillehoj and Chung, 1992). In the lamina propria there are increases in the proportion of lymphocytes expressing TCR$\alpha\beta_1$ (Lillehoj and Chung, 1992). The lamina propria of 1-day-old chicks is poorly developed, containing little stroma (capillaries, lacteals, reticular and muscle fibres) and few lymphocytes (Yason *et al.*, 1987). By 17 days after hatching the lamina propria has changed significantly with increases in stromal tissue, lymphocytes, mononuclear cells and eosinophils.

Cells of the innate immune system develop earlier than T and B cells, with large numbers of heterophils and monocytes being present in the blood at hatching (Burton and Harrison, 1969; Wells *et al.*, 1998). During the first 2 weeks after hatching there are further increases in polymorphonuclear cells in all parts of the intestine (Bar-Shira and Friedman, 2005).

DEVELOPMENT OF IMMUNE RESPONSES TO MODEL ANTIGENS

Systemic immune responsiveness to immunization with bovine serum albumin (BSA) differ following *in ovo* (16 or 18 EID) compared to sub-cutaneous immunisation at 1, 7 or 12 days after hatching; (Mast and Goddeeris, 1999). Antigen-specific IgM and IgY were detected only in those chicks immunized 12 days after hatching, peaking at 7 (IgM) and 10 (IgY) days post-immunization. Chickens immunized at 7 and 10 days were capable of an anamnestic response after a second immunization, however those immunized at 1 day old were not. These data suggest that young chicks (<1 week old) are not capable of mounting an effective antibody response to antigen. Similarly, oral administration of BSA or cloacal administration of haemocyanin only evoked a specific antibody response in chicks older than 8 days of age (Bar-Shira *et al.*, 2003). Other studies by Klipper *et al.* (2001, 2004) demonstrated that orally administered antigens induced systemic tolerance in chicks less than 3 days of age. Klipper *et al.* (2004) hypothesized that the presence of maternal antibodies at hatching may help prevent tolerance against antigens derived from pathogens. They suggested that maternal antibodies block interactions between the immune cells involved in generating tolerance and pathogen-derived antigens. The progeny of hens immunized with BSA were compared with those from non-immunized hens and their capacity to produce BSA-specific antibodies measured after oral immunization at day 1–6 post-hatching with an oral boost at 2 weeks of age. Chicks with high levels of maternal anti-BSA antibodies produced high levels of endogenous anti-BSA antibodies after immunization whereas the progeny of non-immunized hens (with no specific maternal antibody) were tolerized by the oral immunization schedule at 1–6 days.

IMMUNITY TO ENTERIC PATHOGENS

The gut is a site that is constantly challenged by pathogens, where a full spectrum of immune responses is induced and active against enteric challenge. When considering the mechanisms of gut immunity it is important to consider the biology of the pathogens in relation to the host tissues. For example, some pathogens localize predominantly in the gut lumen and cause little damage (e.g. nematode, cestodes and some enteric bacteria) but can persist for extended periods, while others reside in intracellular niches and can cause acute disease (e.g. *Eimeria* spp.). Other pathogens use the intestine as a portal of entry into the tissues and, although residing in the gut for relatively short periods of time, the local mechanisms that limit entry into deeper tissues can be very important. Responses which control pathogens, such as *Salmonella enterica* serovars Gallinarum or Pullorum, will include those active at the gut and in systemic sites. When considering the immune mechanisms that operate to limit infection there are certain commonalities which need consideration. Firstly, there are constitutive barriers to infection (e.g. mucous layers), the composition of which may also change as a consequence of inflammation. To induce an inflammatory response the host first has to detect the presence of a pathogen

(and to discriminate between pathogen and commensal) and many cell types in the gut express PRR including the TLR (Iqbal *et al.*, 2005a). PRR expression is not only restricted to immune cell types; the role of stromal cells in pathogen detection should not be overlooked. Once alerted, a wide range of responses are induced in the gut with combined activities of innate and adaptive cell types resulting from infectious challenge.

The infiltration of heterophils often accompanies the early phase of infection in the gut (e.g. Kogut, 2002; Van Immerseel *et al.*, 2002) and these cells can both kill invading pathogens and contribute to recruitment of other immune cell types. Indeed, heterophils are well equipped for pathogen detection expressing a wide spectrum of TLR (Kogut *et al.*, 2005a). Similarly, NK cells, macrophages and TCR$\gamma\delta^+$ T cells often play a significant role early in infection, limiting establishment and spread of infection. Later during infection, TCR$\alpha\beta^+$ T cells and B cells are activated and infiltrate the lamina propria and IEL compartments; cell types of the adaptive immune system are often involved in the resolution of infection. However, infectious challenge often induces the activity of responses that are ineffective at controlling infection and at times these can be damaging to the host's tissues. Unfortunately, detecting a response does not indicate effectiveness or involvement in controlling infection and "response" data should be interpreted with considerable care.

In the following sections we will describe some of the general concepts in gut immunity with reference to different pathogens in the chicken. As already pointed out, our knowledge of immunity to enteric infection in the chicken is dominated by studies on a very restricted range of pathogens, most notably the enteric *S. enterica* serovars and the protozoan parasites of the genus *Eimeria*. A full understanding of the mechanisms of immunity in the gut will only be achieved after more extensive studies on other infections, including AI and the gut helminths.

Development of Immunity to Enteric Pathogens

The gut is a dynamic immune compartment and substantial development occurs after hatching (as discussed above), hence the mechanisms which operate to limit infection can be very different in young chicks compared with older chickens. The enteric immune system of the young chick is poorly developed and matures rapidly up to 4–6 weeks after hatching.

Perhaps the most dramatic example of age-dependent development of resistance is that seen with the enteric serovars of *S. enterica* (Enteritidis and Typhimurium). Infection of young chicks (<3 days) leads to a severe systemic disease and high mortality, whilst infection is largely restricted to the gut in older chickens, which do not display clinical signs (Gast and Beard, 1989; Barrow *et al.*, 1998). The dramatic differences in infection biology reflects the development of gut immune competence during the first week after hatching (Uni *et al.*, 2000; Geyra *et al.*, 2001).

S. enterica serovar Typhimurium (ST) has also been used to define more subtle changes in gut immune competence that occurs between 1 and 6 weeks after hatching. Oral challenge of chicks at 1, 3 or 6 weeks did not result in overt clinical signs but the time taken to clear enteric infection differed markedly (Beal *et al.*, 2004b). Indeed, irrespective of the timing of challenge all three groups of chicks cleared infection at about the same age, around 9–11 weeks, which suggests that mechanisms operating to clear enteric *Salmonella* develop over a considerable period of time. Interestingly, chicks inoculated at 1 week old also had reduced antigen-specific antibody and T cell proliferation compared with those infected at 3 or 6 weeks of age and were less well protected against re-challenge infection at 15 weeks. Moreover, the influence of host genetics on the time taken to clear infection in 6-week-old chickens was not evident with 10-day-old chicks, which had much weaker T cell, antibody and cytokine responses (Beal *et al.*, 2005). These results have implications for transmission of salmonellosis to humans via contamination of meat from broiler chickens, and the likely success of vaccines applied to young chicks.

Age-dependent increase in resistance is also evident with reovirus infection which correlated with the capacity to produce antigen-specific antibody responses (Mukiibi-Muka and Jones, 1999).

Chicks infected on day of hatch were incapable of producing significant virus-specific IgA, contrasting with the more resistant 3-week-old chickens which produced a substantial IgA response. Age-dependent resistance is less evident in other infection models, for example with the intracellular protozoan, *Eimeria tenella* (Lillehoj and Chai, 1988).

MATERNAL ANTIBODY AND PROTECTION OF THE YOUNG CHICK

The maternal/progeny relationship is important in the protection of young animals particularly against endemic infections. With mammals the transfer of maternal antibodies occurs by both transplacental and colostral routes but with birds all antibodies are transferred via the egg. Both IgM and IgA are secreted into the albumen and can be taken up by the developing chick but IgY is the major maternal antibody transferred via the egg yolk (see Chapter 6). High titres of specific IgY are deposited into the yolk of the developing egg and have a transient, but essential, role protecting chicks from pathogens such as: *Eimeria*, *Cryptosporidium*, *Salmonella* and *Campylobacter* (Rose, 1972; Smith, N.C. *et al*., 1994; Hassan and Curtiss, 1996; Hornok *et al*., 1998; Sahin *et al*., 2003). In challenge experiments, when the progeny of immunized, or recently infected, hens were compared with the progeny of control hens, the level of infection was reduced. The importance of maternal antibody declines with the age of the chick and is considered most important during the first 3 weeks of age. Transfer of protective antibody into the yolk requires high levels of circulating antibody in the hen and naturally occurring maternal protection is probably limited to those infections currently in the environment at the time of lay. For example, the transitory nature of maternal protection was evident with *E. maxima* where significant protection occurred only in chicks obtained from the eggs laid between 17 and 30 days post-infection (Rose, 1972). Nonetheless, the maternal protection route has been exploited in the control of coccidiosis (Smith, N.C. *et al*., 1994) and a commercial vaccine (CoxAbic®) has been developed, based on the maternal transfer concept. The potential for exploiting maternal immunization is attractive in many ways, since large numbers of offspring can be protected by intensive immunization of a relatively few hens. Although this route of immunization deserves further exploitation, protection is transient and maternal transfer of antibody (either from natural ongoing infection or immunization) may also interfere with direct immunization of the chick.

VIRAL INFECTIONS OF THE GUT

Unfortunately there is a paucity of data on the immune mechanisms which operate against enteric viral infections of chickens, although, antigen-specific antibody is readily detected after infection with reovirus and AI (Madeley *et al*., 1971; Brugh *et al*., 1979; Chambers *et al*., 1988; Hunt *et al*., 1988; Taylor *et al*., 1988; Hinshaw *et al*., 1990; Mukiibi-Muka and Jones, 1999; Cardona *et al*., 2006). Evidence for upregulation of a wide variety of immune responses in the lung and spleen was obtained in a recent microarray-based analysis of tissues from naïve chickens and those immunized with the H9N2 strain (Degen *et al*., 2006). AI viruses replicate readily in the gut of chickens and faecal contamination of the environment is likely to be important in transmission of infection. The relative importance of gut and lung tissues in the immunobiology of AI has not been established but gut immune responses induced by exposure to low pathogenicity viruses may be important in protecting chickens from the lethal effects of high pathogenicity strains. The AI virus is a member of the Orthomyxoviridae, a family of enveloped, segmented, single-stranded RNA viruses that are sub-typed according to their expression of serologically distinct haemagglutinin (HA) and neuraminidase (N) surface proteins (for a general information see Horimoto and Kawaoka, 2005; Swayne and Pantin-Jackwood, 2006; Webster *et al*., 2006a). A wide variety of vaccine strategies has been used to protect chickens against AI and many of these prevent the pathological consequences of infection but are less

effective at preventing infection itself or, more importantly, transmission (e.g. Brugh *et al.*, 1979; Chambers *et al.*, 1988; Hunt *et al.*, 1988; Taylor *et al.*, 1988; Robinson *et al.*, 1993; Crawford *et al.*, 1999; Rimmelzwaan *et al.*, 1999; Kodihalli *et al.*, 2000; Tian *et al.*, 2005; Cardona *et al.*, 2006; Gao *et al.*, 2006; Mingxiao *et al.*, 2006; Park *et al.*, 2006; Qiao *et al.*, 2006; Toro *et al.*, 2007; Webster *et al.*, 2006b). The risks associated with partial protection with a vaccine (or by natural exposure to a low pathogenicity virus) occur at both the individual bird and at flock level. These risks include (1) the selection of immune escape variants and (2) environmental contamination with high pathogenicity virus, both of which can be devastating to the overall effectiveness of AI control strategies (Savill *et al.*, 2006; Webster *et al.*, 2006a).

The activity of avian type I IFN in limiting viral replication is well documented (Schwarz *et al.*, 2004; Koerner *et al.*, 2007) and likely to be important in the very earliest stages of infection. Indeed, with many high pathogenicity strains an overwhelming infection of broad tissue specificity develops very rapidly and death of the chicken can occur within 3 days. The adaptation of high pathogenicity isolates to different avian species may be due in part to the ability of these viruses to evade innate immune mechanisms, including the activity of type I IFN (Marcus *et al.*, 2005; Li *et al.*, 2006; Cauthen *et al.*, 2007).

Pathogen recognition is crucial to the host response to infection and examination of the avian TLR repertoire revealed intact functional TLR3, TLR4 and TLR7 (all implicated in recognition of viral pathogen-associated molecular patterns (PAMPs); see Chapter 7 for detailed descriptions) but that TLR8 is disrupted and entirely non-functional in galliform birds (Philbin *et al.*, 2005). Although chTLR7 is functional, exposure of chicken splenocytes or the HD11 macrophage-cell line to TLR7/8 agonists failed to reproducibly elevate production of type I IFN mRNA (Philbin *et al.*, 2005). However, IFN activity was detected (using an antiviral assay) when the imidazoquinolinamine immunoenhancer S28828 (structurally related to R848) was administered orally to chickens, which may indicate the importance of specific cell types in the avian TLR7-induced IFN response (Karaca *et al.*, 1996). In contrast to the TLR7 agonists, exposure of chicken splenocytes to the TLR3 agonist, synthetic double-stranded RNA (polyinosinic-cytidylic acid) induced significant upregulation of IFNα and IFNβ mRNA (Philbin *et al.*, 2005).

The activity of antibody against AI is well established with high-titre antibody responses detected against the HA and N proteins which inhibit viral entry into cultured cells (Madeley *et al.*, 1971; Brugh *et al.*, 1979; Chambers *et al.*, 1988; Hunt *et al.*, 1988; Taylor *et al.*, 1988; Hinshaw *et al.*, 1990; Cardona *et al.*, 2006). The importance of anti-HA antibody-mediated immunity was evident by the abrogation of immunity (generated by an HA-vaccinia vaccine construct) after chemical bursectomy and the ability to passively transfer protective antibody to naïve recipients (Chambers *et al.*, 1988). Although antibody is clearly an important mechanism for limiting viral infection it is often insufficient to completely protect against viral replication. Indeed, in many immune responses to viruses both $CD4^+$ and $CD8^+$ $TCR\alpha\beta^+$ T cells are important for controlling infections by mechanisms that include: help for B cell production of antibody, production of antiviral cytokines (e.g. type I and type II IFN) and direct cytotoxicity of virus-infected cells. The importance of T cells has been best demonstrated with studies of heterologous immunity between H9N2 and H5N1 viruses (Seo and Webster, 2001; Seo *et al.*, 2002). Adoptive transfer of $CD8^+$ T cells from H9N2-vaccinated birds protected recipient birds challenged with H5N1 virus in terms of survival and reduced viral load. By contrast $CD4^+$ T cells, B cells or cells from naïve donor chickens were unable to confer immunity (Seo and Webster 2001). Using a depletion protocol with monoclonal antibodies TCR1 ($TCR\gamma\delta$), TCR2 ($TCR\alpha\beta_1$) and TCR3 ($TCR\alpha\beta_2$) and immunization with H9N2, only the anti-TCR2 treatment resulted in an exacerbated infection after challenge with H5N1 (Seo *et al.*, 2002). Similarly anti-CD3 or CD8 treatment, but not anti-CD4 treatment, exacerbated challenge infection (Seo *et al.*, 2002) and IFNγ-containing $CD8^+$ T cells were detected in the spleen and lungs of the H9N2 immunized birds. The impact of the high pathogenicity AI virus H5N1 has stimulated considerable interest in understanding the AI immunology in the chicken. We particularly look forward to the impact of these studies on fundamental avian gut immunology.

BACTERIAL INFECTIONS OF THE GUT

Salmonella

The outcome of infection with *S. enterica* differs according to: serovar, chicken age, history of exposure to salmonellae and, to a lesser degree, chicken genotype. Challenge with the highly host-restricted serovars, Gallinarum and Pullorum causes more typhoid-like diseases in chickens of any age and with considerable mortality. In contrast, infection with broad host range serovars, Typhimurium and Enteritidis results in a long-lived infection that is generally limited to the gut in chickens greater than 2–3 days of age. However, with young chickens both of these enteric serovars cause substantial mortality with high numbers of bacteria replicating in the systemic organs.

Both *S.* Gallinarum and Pullorum are aflagellate and thought to enter systemic sites via enterocytes and lymphatic tissues such as the PP and CT (Barrow and Duchet-Suchaux, 1997). Once they cross the gut these serotypes are carried to systemic sites, probably within cells of the reticulo-endothelial system, and can then be isolated from sites such as the liver, spleen, ovaries and bone marrow. Very little inflammation occurs in the gut after infection with *S.* Pullorum, where there is little heterophil infiltration (Henderson *et al.*, 1999). Immune responses to these serovars are characterized by high titres of specific antibodies and T cell proliferation (Wigley *et al.*, 2005). Those chickens that survive the acute phase of *S.* Pullorum infection remain persistently infected with low numbers of salmonellae present in splenic macrophages (Wigley *et al.*, 2001). Hormonal changes at point of lay may lead to a substantial transient depression in immune responsiveness and recrudescence of infection (Wigley *et al.*, 2005). Differential resistance/susceptibility of chicken lines to systemic salmonellosis has allowed identification of host loci associated with disease outcome. The *SAL1* locus has been mapped to chicken chromosome 5 and is thought to be involved in survival of salmonellae within macrophages (Mariani *et al.*, 2001; Wigley *et al.*, 2001). Additional candidate loci involved in the resistance to systemic salmonellosis in the chickens include regions containing the MHC, *Nramp1* and TLR4 (Hu *et al.*, 1997; Mariani *et al.*, 2001; Liu *et al.*, 2002; Leveque *et al.*, 2003). In contrast, genetic resistance of chickens to enteric colonization with *S.* Typhimurium and Enteritidis does not associate with the *SAL1* or MHC loci (Barrow *et al.*, 2004). Evidence for acquired immunity to challenge with *S.* Gallinarum led to the development of a live-attenuated vaccine strain known as 9R, which is used to control fowl typhoid in countries where biosecurity measures have failed to control disease. A recent report has indicated that the cross-protection with an attenuated live *S.* Enteritidis vaccine TAD *Salmonella* Vac® E (Lohmann Animal Health) may also protect against virulent *S.* Gallinarum challenge (Chacana and Terzolo, 2006).

Enteric salmonellosis is caused by colonization of the chicken gut with *S.* Typhimurium and *S.* Enteritidis. The drive to control enteric salmonellosis is because of the ability of these serovars to infect and cause disease in humans as a food-borne zoonosis following contamination of chicken meat products and eggs. In chicks aged more than a few days old these serovars colonize the gastrointestinal tract (predominantly ileum and caecum), where they can persist for many weeks with few bacteria invading the epithelium and entering systemic sites such as the spleen and liver (Barrow *et al.*, 1987). Indeed, although there is an early transient inflammatory reaction in the gut, lesions are microscopic and clinical disease is only associated with infection of chicks less than 3 days old. Age-associated changes in disease susceptibility are well defined for the enteric *Salmonella* serovars (discussed in detail above) and probably reflect the development of gut immune competence. Such changes first reduce bacterial translocation and restrict the majority of salmonellae to the gut with the rate of clearance from the gut increasing in older birds. Contamination of eggs with *S.* Enteritidis is thought to be associated with its prolonged survival in systemic sites, translocation to the oviduct occurring around the onset of lay, which may be associated with transient immunosuppression as reported for *S.* Pullorum (Wigley *et al.*, 2005).

In the gut, early host responses to infection include a localized inflammatory response with an associated influx of heterophils (Kogut *et al.*, 1994). Interestingly, a role for TLR5-flagellin

interactions in restricting *S.* Typhimurium to the gut lumen was proposed after more rapid translocation to systemic sites was observed with an aflagellular mutant *fliM S.* Typhimurium (Iqbal *et al.*, 2005b). Despite greater uptake, the aflagellate salmonellae induced much less IL-1β and IL-6 in the gut and a reduced heterophil infiltrate (Iqbal *et al.*, 2005b). A wide range of cell types (heterophils, macrophages, B cells fibroblasts and epithelial cells) respond strongly to exposure to live salmonellae or products of this bacterium including the well-defined TLR agonists lipopolysaccharide, flagellin and CpG-containing DNA (Kaiser *et al.*, 2000; Dil and Qureshi, 2002; Iqbal *et al.*, 2005b; Kogut *et al.*, 2005b, 2006, 2007). Live salmonellae readily invade cultured heterophils and macrophages but are then killed by these cells, a response enhanced by prior exposure of cells to pro-inflammatory cytokines such as IFNγ or IL-2 (Kogut *et al.*, 2002, 2003). The level of responsiveness of heterophils and macrophages to *Salmonella* or their extracts correlates with the level of susceptibility of chickens and this has led to the proposal that these cells are, in some way, involved in protection (Dil and Qureshi, 2002; Swaggerty *et al.*, 2003, 2004).

Chickens infected with enteric *Salmonella* serovars mount a substantial immune response typified by high levels of antigen-specific antibodies (IgM, IgY and IgA) (reviewed in Zhang-Barber *et al.*, 1999), strong T cell responses (Beal *et al.*, 2004a, 2005) and increases in expression of mRNA in the spleen and gut encoding an array of cytokines and chemokines (Beal *et al.*, 2004a, 2005; Withanage *et al.*, 2005). Evidence that the host response controls infection includes the age-dependent ability to clear the *Salmonella* from the gut and the more rapid clearance after secondary challenge (Barrow *et al.*, 1990; Beal *et al.*, 2004a, b). The timing of clearance in 6-week-old chickens correlates with the peak antibody and T cell responses and, considering the luminal location of the *Salmonella*, the specific IgA response is a prime candidate effector mechanism. However the role of B cells and antibodies in protecting the chicken against enteric *Salmonella* infections is less clear cut. Desmidt *et al.* (1998) rendered chickens B cell deficient by a combination of cyclophosphamide and testosterone proprionate treatments and concluded that the prolonged persistence of *S.* Enteritidis in these birds indicated a prominent role for B cells in the clearance phenotype. However, more recent studies comparing different methods of producing B cell-deficient chickens, demonstrated that surgical bursectomy at EID17 had no effect on the capacity of chickens to clear either a primary or secondary *S.* Typhimurium infection (Beal *et al.*, 2006a). By contrast chickens treated with cyclophosphamide post-hatch (usually termed chemical bursectomy) and challenged at 6 or 15 weeks of age took longer to clear a primary infection. These data suggest that a cyclophosphamide-sensitive (and non-recoverable), non-B cell mechanism is responsible for clearance of the primary infection. Extensive literature on cyclophosphamide-treated chickens indicates a transient suppression of non-B cell compartments with recovery by ~4 weeks post-treatment (Linna *et al.*, 1972; Rouse and Szenberg, 1974; Sharma and Lee, 1977; Ficken and Barnes, 1988; Corrier *et al.*, 1991; Marsh and Glick, 1992; Arnold and Holt, 1995; Mast *et al.*, 1997; Russell *et al.*, 1997; Kim *et al.*, 2003). These data with *Salmonella* suggest one or more, as yet unidentified, mechanism(s) is involved, and important for mediating clearance. Interestingly, both surgically bursectomized chickens and cyclophosphamide-treated chickens were capable of clearing a secondary infection at the same rate as intact chickens. These data suggest that either different non-B cell mechanisms are involved in the more rapid clearance evident after secondary challenge or that primary infection stimulates the rescue of an effector mechanism in cyclophosphamide-treated chickens. The lack of a requirement for B cells and antibody in enteric salmonellosis must be considered surprising and highlights the need for careful interpretation of the role for immune mechanisms, based on a detected response.

The role for T cell responses in clearance of enteric salmonellae has not been proven but in the absence of an essential role for B cells and with faster clearance of infection at secondary challenge (evidence for immune memory) these responses are likely to be important. Cellular responses induced by infection include antigen-specific delayed-type hypersensitivity responses and changes in the distribution of T and B cells, antigen-specific T cell proliferation and T-dependent

class switching in B cells (Lee *et al.*, 1983; Berndt and Methner, 2001; Beal *et al.*, 2004a, b, 2005). Strong cellular and humoral immune responses correlated temporally with clearance of *S.* Typhimurium from the gut following primary infection, although these responses were less intense following re-challenge (Beal *et al.*, 2004a, b). Immediately prior to clearance of primary infection significant increases in mRNA encoding IL-1β, IFNγ and TGF-β were observed. The global gene expression profiles of the gut in two lines of chicken with differential susceptibility to infection revealed upregulation of a wide range of mRNA, in particular some suggesting pronounced T cell infiltration or activation such as ZAP70 (Van Hemert *et al.*, 2006). Chickens have relatively high numbers of TCR$\gamma\delta^+$ T cells and these clearly respond after oral infection with *S.* Enteritidis, especially with those expressing CD8 (either as a CD8$\alpha\alpha$ homodimer or CD8$\alpha\beta$ heterodimers; Berndt *et al.*, 2006). A range of responses were assessed using inbred chicken lines which differed in susceptibility to infection at 6 weeks of age but not at 10 days old, only the level of antigen-specific T cell response correlated with the rate of clearance in both (Beal *et al.*, 2005). Various vaccination strategies have been employed with *S.* Enteritidis and Typhimurium including administration of heat killed and various live attenuated strains (reviewed in Van Immerseel *et al.*, 2005). While successful the level of immunity does not match that induced by previous exposure to wild-type salmonellae (Barrow *et al.*, 1990). Priming with either *S.* Typhimurium or *S.* Enteritidis primes a strong cross-reactive T and B cell response and considerable protection to challenge with either serovar (Beal *et al.*, 2006b) suggesting conservation of protective antigens.

Campylobacter

Campylobacter spp. are currently the most common causative agents of bacterial human foodborne disease (particularly *C. jejuni*); however, in the chicken they do not cause disease and are proposed to act as commensals (Beery *et al.*, 1988). Morphologically, *Campylobacter* spp. are Gram-negative rods, which are either curved, spiral or "S" shaped, with uni- or bi-polar flagella making them highly motile. *Campylobacter* are microaerophilic and thermophilic bacteria that colonize the mucous layer, most commonly in the caecum, where they can be found at up to 10^9 cfu/g (Beery *et al.*, 1988; Cawthraw *et al.*, 1996; Dhillon *et al.*, 2006). Although *C. jejuni* are predominantly found in the gut of chickens small numbers have been isolated from both primary and secondary lymphoid organs (Cox *et al.*, 2005, 2006). Natural infection of chickens often occurs at about 2 weeks of age, concurrent with reducing amounts of maternal antibody (Sahin *et al.*, 2003). Transmission between chickens is thought to occur horizontally, with little or no vertical transfer (Lee and Newell, 2006). With most strains of *Camplyobacter* colonization is long lived, although there are differences in the level of colonization associated with host genetics (Boyd *et al.*, 2005).

Very few studies have examined the host response to *Campylobacter* infection in the chicken; of these most have concentrated on vaccination using inactivated cultures or by measuring *Campylobacter*-specific antibody production. Vaccination with formalin-inactivated *C. jejuni* was reported to afford a modest degree of success in experimental trials (Rice *et al.*, 1997) but the small reductions in bacterial load in the caeca or the small increase in specific antibody were not statistically significant compared with unvaccinated controls. Significant increases in antigen-specific IgA antibodies were detected in the gut and serum following infection with *C. jejuni* (Myszewski and Stern, 1990; Stern *et al.*, 1990; Cawthraw *et al.*, 1996). Significant responses were directed against the flagellin and outer membrane proteins as revealed by western blot analysis. Biliary antibodies were purified and when added to the inoculating dose reduced the ability to initiate colonization (Stern *et al.*, 1990) indicating that IgA can have a detrimental effect on *C. jejuni*. The production of high levels of antibody indicates that the host is responding to infection and therefore must be inducing a pro-inflammatory response greater than that expected from commensal organisms. Exposure of cultured chick kidney cells or the macrophage-like cell line HD11 to *C. jejuni* led to increased levels of nitric oxide and pro-inflammatory cytokine production (Smith *et al.*, 2005).

Necrotic Enteritis

Necrotic enteritis (NE) lesions in chickens are often associated with *Clostridium perfringens*, a bacterium naturally found in the gut of 75–90% of poultry. High clostridial load does not directly correlate with NE incidence and disease is influenced by factors such as co-infection, intestinal pH and diet (Williams, 2005). Pathogens that damage the small intestine, such as *E. maxima* and *acervulina* are thought to provide an environment suitable for differentiation and proliferation of *C. perfringens* (Van Immerseel *et al.*, 2004). *C. perfringens* is a Gram-positive anaerobic bacterium that can produce toxins and enzymes that cause enteric lesions. The strains of *C. perfringens* can be classified by the toxins that they produce into 5 groups (A–E). Only strains A and C are associated with disease in the chicken (Songer, 1996). The α toxin (produced by A and C strains) is a phospholipase C sphingomyelinase that hydrolyses phospholipids leading to disruption of the host cells membrane and stimulation of the arachidonic cascade which stimulates production of inflammatory mediators (Songer, 1997). The precise mechanism of action of the β toxins (produced by strain C) is still unknown although they are also thought to disrupt cell membranes.

Earlier studies successfully experimentally induced acute NE by infecting chickens with *Eimeria* and then feeding them feed contaminated with *C. perfringens* (Al-Sheikhly and Al-Saieg, 1980) or by infecting with *C. perfringens* broth cultures (Al-Sheikhly and Truscott, 1977b). Oedema, dilation of the surrounding blood vessels and desquamation of epithelial cells in the gut mucosa occured rapidly (Al-Sheikhly and Truscott, 1977c). Within 3 h the mucosa turned greyish and became thickened, a marked oedema and a detachment of the epithelial layer from the lamina propria were apparent. Necrosis of the epithelial layer and lamina propria is evident by 5 h, with congested blood vessels and an infiltration of both mononuclear cells and heterophils. By 8–12 h there was massive necrosis of the villi with necrotic zones reaching down to the crypts and changes in the liver, heart, kidney and bursa.

The sub-clinical form of the disease can be initiated experimentally by infecting birds with *C. perfringens* cultures that have been washed and re-suspended in PBS (Al-Sheikhly and Truscott, 1977a). By comparison infecting chickens with either bacteria isolated from broth, or the broth itself, usually results in the acute form of disease. The sub-clinical disease usually affects the gut only in localized foci and may be associated with hepatitis and cholangiohepatitis (Lovland and Kaldhusdal, 1999).

Very few studies have examined the host immune response or the capacity to vaccinate against disease, probably due to the difficulty in reproducing the disease under experimental conditions. However, a recent study conducted by Thompson *et al.* (2006) investigated the use of a wild-type and attenuated *C. perfringens* to protect against a wild-type re-challenge. Chickens immunized with either wild-type virulent strains or strains that were deficient in the α-toxin demonstrated lower mean lesion scores following a wild-type challenge than the unvaccinated controls. Whilst these results demonstrate the capacity for chickens to mount immune responses that protect against NE, the components underpinning the protective immune response are still not understood.

PARASITIC INFECTIONS OF THE GUT

A wide range of protozoan and helminth parasites infect the gut of the chicken, the most important of these for the poultry industry being the intracellular protozoan parasites of the genus *Eimeria*. Many poultry production practices have limited the impact of other parasitic diseases but with increased free-range farming these are likely to become more prevalent.

Eimeria spp.

Infection with the eimerian parasites causes enteric coccidiosis which results in a wide spectrum of disease, dependent on the site of infection, age and strain of chicken, and the biology of

the infecting *Eimeria* spp. Chickens are challenged by seven species of *Eimeria* with the most important being *tenella*, *maxima*, *necatrix* and *acervulina*. The biology of eimerian infections has been reviewed extensively, including immunity and vaccination strategies (e.g. Ovington *et al.*, 1995; Lillehoj and Trout, 1996; Smith and Hayday, 1998; Vermeulen, 1998; Lillehoj and Lillehoj, 2000; Shirley *et al.*, 2005; Dalloul and Lillehoj, 2006). In this section the discussion will be restricted to the major mechanisms of immunity and for more comprehensive coverage of the topic of anti-eimerian immunity the reader is encouraged to refer to the reviews indicated above.

The eimerian life cycle involves oral acquisition of infection, serial invasion of enterocytes by the zoite stages, development of stages within cells (schizonts and gametocytes), fertilization and release of oocysts into the environment. The most important features of the life cycle with reference to immunity are the phases of intracellular development (avoiding the action of antibody), the short duration of the infection cycle (meaning that any immune effect must be rapid) and the level of immunity generated by primary exposure to small numbers of parasites (hence vaccination works). Immune mechanisms operate to limit the magnitude of primary infection and, depending on the *Eimeria* spp. and immunization/challenge schedule, can completely prevent oocyst production at secondary challenge.

The specificity of protection induced by prior exposure is restricted to the *Eimeria* spp. used to prime the birds (reviewed in Rose, 1987) and can be strain specific with some parasites, such as *E. maxima* (e.g. Martin *et al.*, 1997; Smith *et al.*, 2002). The level of immunity conferred by primary infection is dependent on the *Eimeria* spp. and is influenced by parasite dose, host age and host genetics (e.g. Lillehoj, 1988; Zhu *et al.*, 2000; Smith *et al.*, 2002). With the most immunogenic *Eimeria* spp. (e.g. *E. maxima* in the chicken or *vermiformis* in the mouse) a very small priming infection leads rapidly to the establishment of, essentially, complete immunity (i.e. no oocysts produced). Priming with similar numbers of less immunogenic *Eimeria* spp. (e.g. *E. tenella* in the chicken or *pragensis* in the mouse) induces substantial immunity to re-challenge infection but some oocysts are produced. Complete immunity to the less immunogenic *Eimeria* spp. can be established by multiple priming infections. In the case of the fully immune host the majority of parasites are killed very rapidly with essentially no parasites remaining after 48–72 h post-challenge (e.g. Riley and Fernando, 1988; Rose *et al.*, 1992b).

Infection of chickens induces a wide variety of responses in the gut including heterophil infiltration, NK cell activation, antibody production, T cell activation and upregulation of many cytokines and chemokines (reviewed in Rose, 1987; Lillehoj and Lillehoj, 2000; Shirley *et al.*, 2005; Dalloul and Lillehoj, 2006). It is clear that many of the responses induced by infection with the *Eimeria* spp. are at best irrelevant to protection and at worst contribute to the damage caused by infection (Rose and Hesketh, 1982b; Roberts *et al.*, 1996). Here we will focus on the responses that have been shown to contribute to protection using studies with avian and murine *Eimeria* spp. to illustrate the types of response that kill the eimerian parasites.

The short duration of the life cycle influences the mechanisms of immunity that can contribute to limiting the magnitude of primary infections and differ according to the *Eimeria* spp. and the genetics of the host. The genetics of the host can affect the level of parasite replication during primary infection with many *Eimeria* spp. (e.g. Rose *et al.*, 1984; Lillehoj and Ruff, 1987; Lillehoj *et al.*, 1989; Bumstead *et al.*, 1995) and differential resistance has been correlated to the rate of immune induction in the gut (Rose *et al.*, 1990; Rothwell *et al.*, 1995; Yun *et al.*, 2000) or to the basal level of IL-10 production (Rothwell *et al.*, 2004).

Mechanistically, T cells have been implicated in limiting primary infections in both murine (Rose and Hesketh, 1982a; Roberts *et al.*, 1996; Smith and Hayday, 2000a, 2000b) and avian hosts (Rose and Long, 1970; Rose and Hesketh, 1982b; Trout and Lillehoj, 1996). With the murine parasite, *E. papillata*, immune-mediated resistance to primary infection is mostly associated with the activity of NK cells (Schito and Barta, 1997) compared with their very minor role with *E. vermiformis* (Smith, A.L. *et al.*, 1994; Rose *et al.*, 1995). The differential roles for NK and T cells in the control of these infections are probably related to the very short duration of the *E. papillata* life cycle which is completed before T cell-mediated immunity can develop.

In adult animals, immunity to *E. vermiformis* (considered a model for *E. maxima* in the chicken) is dependent on the rapid activity of $CD4^+$ $TCR\alpha\beta^+$ T cells restricted by MHC class II and mediated by the production of IFNγ (Rose *et al.*, 1988, 1989, 1991b, 1992a; Roberts *et al.*, 1996; Smith and Hayday, 2000b). However, in the absence of $TCR\alpha\beta^+$ T cells the $TCR\gamma\delta^+$ T cell population can mediate a protective effect (Smith and Hayday, 2000a); this subset of cells that can be important in young animals (Ramsburg *et al.*, 2003). In chickens, the numbers of $TCR\gamma\delta^+$ T cell IEL are altered by infection suggesting a response but the role for these cells has not been defined (Lillehoj, 1994; Bessay *et al.*, 1996; Hong *et al.*, 2006). Partial depletion of $CD4^+$ T cells by administration of monoclonal antibody against the CD4 antigen prior to primary challenge led to exacerbation of infection with *E. tenella,* but not *E. acervulina*, indicating that the mechanisms of resistance to primary infection may differ between the avian *Eimeria* spp. (Trout and Lillehoj, 1996), as has been reported for the murine parasites (see above). Depletion of $CD8^+$ T cells led to a decreased oocysts output with both *E. tenella* and *acervulina* (Trout and Lillehoj, 1996) and this was attributed to depletion of cells involved in transport of sporozoites.

With both *E. vermiformis* and *E. maxima* there seems to be little requirement for B cell activity or antibodies in control of primary infection (Rose and Hesketh, 1979; Rose *et al.*, 1988; Smith and Hayday, 2000b). However, it is possible to interfere with parasite invasion by administration of large amounts of specific antibody (Rose, 1971) and this has led to the development of a maternal antibody-based approach to protect chicks by immunization of hens (Wallach *et al.*, 1995).

Infection of chickens with *Eimeria* spp. induces upregulation of numerous cytokines in the intestine but only IFNγ has been shown to mediate an anti-eimerian effect. Using anti-IFNγ treatment or IFNγ knockout mice, a prominent role for IFNγ in limiting infection has become well established with murine *Eimeria* spp. (Rose *et al.*, 1991b; Schito and Barta, 1997; Smith and Hayday, 2000a). Treatment of cultured cells with recombinant IFNγ (from the appropriate host species) prior to infection with various *Eimeria* spp. inhibits sporozoite invasion and subsequent development (Kogut and Lange, 1989a, b; Rose *et al.*, 1991a; Lillehoj and Choi, 1998). Moreover, administration of recombinant chicken IFNγ reduced the magnitude of infection *in vivo* (Lillehoj and Choi, 1998).

Primary infection with all *Eimeria* spp. leads to substantial immunity to secondary challenge and the fully immune host terminates infection very rapidly. In the immune animal the intracellular sporozoite represents the main target for immunity (reviewed in Rose, 1987; Shirley *et al.*, 2005) although where the level of immunity is insufficient to kill all invading parasites it is likely that immune-mediated attrition of parasites occurs throughout the life cycle. Mechanistically, it is clear that $TCR\alpha\beta^+$ T cells are essential for immunity (Smith and Hayday, 2000b) and studies with athymic mice and rats indicate that these cells are thymus dependent for their development (Rose and Hesketh, 1979; Rose *et al.*, 1984). With *E. vermiformis*, where the host is completely immune to secondary challenge, it is clear that there is substantial redundancy in the requirement for immunity and many immunodeficient strains of mice are completely, or near completely, immune (Smith and Hayday, 2000b). Mice deficient in all $TCR\alpha\beta^+$ T cells (i.e. $TCR\beta^{-/-}$) remain highly susceptible to infection, whereas those deficient in MHC class II expression produce few parasites and those deficient in MHC class I expression ($\beta 2m^{-/-}$ or $TAP1^{-/-}$) or IFNγ produce no oocysts (Smith and Hayday, 2000b). Treatment with monoclonal antibodies against CD4, CD8α or IFNγ had no, or little, impact on immunity after infection with *E. vermiformis* (Rose *et al.*, 1989, 1992a). Very low numbers of oocysts were produced after anti-CD8α treatment of *E. vermiformis* immune mice (Rose *et al.*, 1992a) and this is similar to experiments in chickens infected with *E. tenella* or *E. acervulina* (Trout and Lillehoj, 1996). Although $TCR\gamma\delta^+$ T cells can mediate protective immunity in young animals in the mouse there is no evidence for the development of immune memory with this cell population (Smith and Hayday, 2000a). With *E. papillata* where primary infection induces partial immunity, secondary challenge of MHC class II deficient mice was exacerbated indicating a role for $CD4^+$ T cells in immunity (Schito *et al.*, 1998). In summary, the mechanisms that serve

to protect the immune or partially immune animal are TCR$\alpha\beta^+$ T cell dependent and involve both $CD4^+$ and $CD8^+$ T cell activities.

Commercially, vaccination is a realistic alternative to the prophylactic use of anticoccidial drugs and is largely based on the use of carefully controlled numbers of wild-type or attenuated parasites (reviewed in Shirley *et al*., 2005). The need to incorporate examples of each species (and multiple strains with *E. maxima*) in live vaccines has driven interest in developing sub-unit vaccine approaches. Some success has been achieved using various recombinant antigens (reviewed in Shirley *et al*., 2005) but the degree of protection is often unsatisfactory. The reasons for this may be due to the inclusion of the wrong antigens or to the delivery systems employed being sub-optimal for protection in the gut. Some studies have used live vector systems such as *Salmonella* to enhance delivery to the gut and others have used DNA vaccination technologies with enhancement by the inclusion of avian cytokines (e.g. Min *et al*., 2001) but more work is needed to further improve the delivery of vaccine antigens. Identification of the appropriate antigen for vaccination is not a trivial task since many of the responses induced by infection are not protective. Over recent years an approach based on parasite genetics with two strains of *E. maxima* has been developed and this has identified that only a few regions of the parasite genome are under selective pressure from the protective immune response (Blake *et al*., 2004). Identification of the antigens encoded in these regions of the genome may help identify more generally the characteristics of eimerian antigens that confer protection. The *Eimeria tenella* genome sequencing project (http://www.sanger.ac.uk/Projects/E_tenella/) is a resource that could impact on future vaccine design and allied with genetic and/or library screening approaches offers real opportunity for rapid progress to be made in coccidiosis vaccine research.

Other Parasitic Infections

A wide range of non-eimerian parasites have been reported to infect the chicken gut including various helminths (e.g. *Heterakis* spp., *Ascaridia* spp.) and other protozoa (e.g. *Cryptosporidium* spp., *Histomonas* sp.). Unfortunately very little work has been published on the immune responses induced by infection and those that mediate protective immunity.

Most descriptions of responses induced by non-eimerian intestinal parasites are with the protozoan *Cryptosporidium baileyi* and the nematode *Ascaridia galli*. Infection with either of these organisms induces a variety of responses including antigen-specific antibodies, lymphocyte proliferation assays and cytokines (Current and Snyder, 1988; Malviya *et al*., 1988; Naciri *et al*., 1994; Sreter *et al*., 1996, 1997; Hornok *et al*., 1998; Degen *et al*., 2005). Infection with *A. galli* induced increased levels of IL-4 and IL-13 mRNA but not IFNγ mRNA in the intestine of chickens at 14 days post-infection (Degen *et al*., 2005) supporting the hypothesis that Th2-biased responses predominate during enteric nematode infections. The effectiveness of host immune response in controlling enteric parasites is evident by the clearance of primary infection and the enhanced clearance or reduced level of infection seen at challenge of previously exposed or immunized individuals (Goyal *et al*., 1984; Current and Snyder, 1988; Malviya *et al*., 1988; Naciri *et al*., 1994; Sreter *et al*., 1997; Hornok *et al*., 1998; Gauly *et al*., 2005). The ability to resolve primary infection and immunity to secondary infection with the intracellular protozoan, *C. baileyi* is dependent on T cells rather than B cells as evident by studies with partially thymectomized and bursectomized chickens (Sreter *et al*., 1996). However, maternally-derived antibody appears to confer partial protection against infection of the progeny of hens immunized by three large doses of parasites (Hornok *et al*., 1998) probably by interfering with zoite invasion.

CONCLUDING REMARKS

Our aim has been to describe the development and function of immune mechanisms in the gut in broad terms, especially with reference to infectious challenge. Unfortunately this has necessitated a selective approach to illuminate the major mechanisms of gut immunity and we recommend

that the reader supplements the information in this chapter by making use of the literature cited. The gut is a complex immunological tissue and the immune mechanisms that operate to protect this site from pathogen challenge are diverse. One important point is that the immune system is a responsive system and the induction of a response does not necessarily indicate a protective effect. It is much more difficult to examine the mechanisms of immune protection than to measure a response, but the entire community needs to focus on developing approaches where functional evidence can support description of the events that occur during infection. Since the broiler chicken is short lived, and most development of the gut immune system occurs shortly after hatching; much avian immunology that is commercially relevant attempts to understand a system in a state of flux. Hence, subtle differences in age, health status, microbial environment or genetics can have profound effects on the mechanisms available for immune protection.

Immune-mediated control of infectious disease by enhanced resistance or by classical vaccination is accepted and well used in the commercial poultry sector. However, induction of immunity in the gut is more difficult than with other systemic sites, often requiring targeting to mucosal surfaces. Indeed, inducing immunity in the gut is affected by chick development, oral tolerance and the immunomodulatory environment within the gut. The most successful enteric vaccines are based on live pathogens or are vectored by live pathogens and a greater understanding of how the gut immune system is stimulated, and which mechanisms operate against specific gut pathogens, will provide the foundation for immune control and vaccination in the future.

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14 THE AVIAN RESPIRATORY IMMUNE SYSTEM

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INTRODUCTION

To perform its primary function, the delivery of oxygen to and the removal of carbon dioxide from the body, the respiratory system continually ventilates the lung. As a consequence, particles and particle-associated micro-organisms are inhaled as unavoidable constituents of the tidal air. Intensive management systems for poultry are frequently associated with high loads of dust and pathogens in the environment and therefore pose a particular stress to the respiratory system (Glisson, 1998; Villegas, 1998). Furthermore, antigens can be intentionally delivered to the respiratory tract in the form of aerosols and this method for vaccination has been shown to be highly effective (see Chapter 20). The respiratory immune system has developed strategies to remove inhaled particles and to adequately respond to those micro-organisms that succeed in crossing the epithelial barrier for maintaining its integrity and functions. Here, we will discuss the major features and functional aspects of the defence system of the avian respiratory tract. Since the avian respiratory system differs significantly from that of mammals, this chapter will begin with a brief introduction to its unique anatomy – for more detailed descriptions the reader is referred to McLelland (1989), King (1993), Powell (2000) and Maina (2002).

Besides the respiratory tract, the paraocular Harderian glands (HG), the conjunctiva-associated lymphoid tissue (CALT) and the paranasal organs come into contact with aerosolized particles and micro-organisms (see Chapter 2). These structures harbour organized lymphoid follicles as well as scattered lymphocytes, indicating that they may play a role in immunoprotection of the head-associated mucosal surface (Rose, 1981). The secretion of the HG lubricates

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the nictitating membranes and is further discharged via the lachrymal ducts into the mouth where it can be swallowed, inhaled or expectorated. In contrast, the lateral nasal gland secretions discharge into the nasal cavity from where they can be inhaled. Via this route antimicrobial factors and secretory antibodies of the HG and nasal glands reach the mucosal surface of the head and trachea.

ANATOMY OF THE RESPIRATORY TRACT

The morphology of the avian respiratory system, with its air sac system, rigid lung structure and the continuous air flow rather than tidal ventilation, is considerably different from that of other vertebrates. The upper airway originates at the nares and at the mouth. During the passage through the nasal cavity, the air passes an expanded surface area, the mucosa-covered conchae which contain organized lymphoid structures called the nasal-associated lymphoid tissue (NALT; Bang and Bang, 1968). Air flow can take either of two pathways: through the nasal or the buccal cavities, continuing to travel through the larynx, which primarily prevents the entry of foreign bodies to the trachea. The trachea that has variable length in different avian species connects the larynx with the syrinx, the organ of voice production in birds. The tracheal surface is covered by a ciliated epithelium that contains mucus-producing goblet cells (Duncker, 1974). Lymphoid follicles and scattered lymphocytes are found in the lamina propria. The two primary bronchi originate from the syrinx. They each have a short extrapulmonary section before entering the lung from the ventromedial aspect and crossing its entire length, before finally opening into the abdominal air sac (Fig. 14.1). From the intrapulmonary section of the primary bronchus a

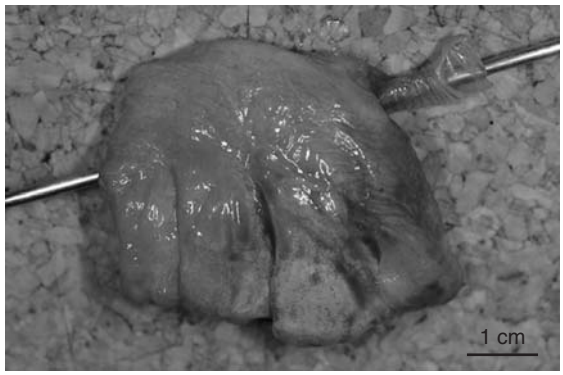

FIGURE 14.1 Right chicken lung with a blunt probe indicating the localization of the primary bronchus. The syrinx with the primary bronchus is shown on the right side.

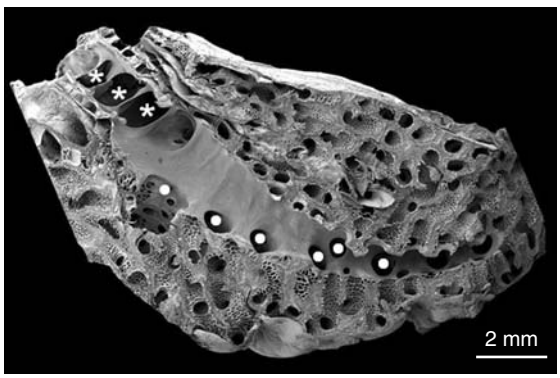

FIGURE 14.2 Longitudinal section of a primary bronchus. Openings to the medioventral (∗) and mediodorsal (•) secondary bronchi are indicated (SEM preparation).

highly organized tubular network branches out and connects to nine air sacs filling most of the residual body cavity not occupied by the other organs.

The avian lung is a relatively rigid structure which does not expand or contract appreciably during the respiratory cycle (Jones *et al.*, 1985). This feature, together with the absence of a diaphragm, indicates that ventilation is accomplished by a strikingly different mechanism compared with that of mammals. Ventilation is achieved by the action of inspiratory and expiratory muscles which increase or decrease the body volume and consequently the volume of the air sacs. Thus, the air sac system acts like a bellows that forces air through the gas exchange tissue during both the inhalatory and expiratory phases of the cycle. During inhalation, air flows through the primary bronchus, bypassing the cranially-located openings of the medioventral secondary bronchi and flows into the caudal air sacs or through the mediodorsal and lateroventral secondary bronchi (Fig. 14.2). This caudal to cranial flow pattern is also evident during expiration (Fig. 14.3) and provides the basis for continuous ventilation and the high efficacy of gas exchange in the avian lung (Powell and Scheid, 1989; Powell, 2000). A functional consequence of this flow pattern is that particles are primarily deposited in the caudal regions of the lung, as was first described for soot deposition in pigeons captured at train stations (Dotterweich, 1930). This observation may explain the absence of organized bronchus-associated lymphoid tissue (BALT) in the cranial part of the lung and its presence at the junctions of the primary bronchus and the caudal secondary bronchi in some avian species, such as the chicken (Fagerland and Arp, 1993b).

From the secondary bronchi, a large number of "tertiary bronchi" called parabronchi originate, which connect the mediodorsal and lateroventral with the medioventral secondary bronchi (Duncker, 1974; McLelland, 1989). They represent the functional unit of gas exchange in the avian lung and are organized in a parallel series separated from each other by a connective tissue which contains large blood vessels and lymphoid follicles (Fig. 14.4). Inhaled air flows through the parabronchial lumen and then centrifugally through the atria and infundibula into the air capillaries (Fig. 14.5). The air–blood capillary interface essentially consists of an epithelium,

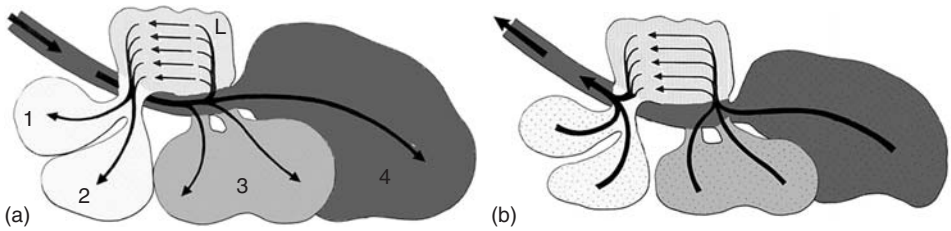

FIGURE 14.3 Illustration of the air flow pattern during (a) inspiration and (b) expiration in the avian lung. L, Lung, 1. clavicular air sac, 2. cranial thoracic air sac, 3. caudal thoracic air sac, 4. abdominal air sac.

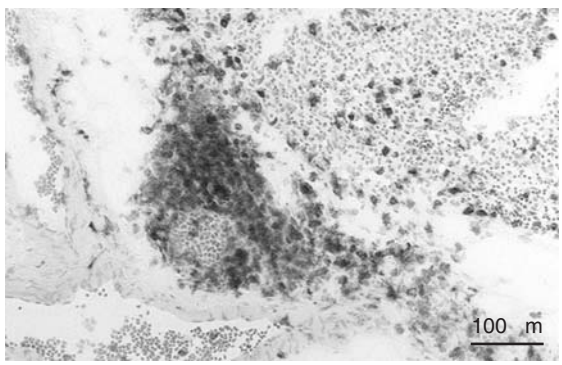

FIGURE 14.4 Organized lymphoid aggregates in the connective tissue between tertiary bronchi. Immunohistological staining with an anti-CD3 monoclonal antibody (CT3).

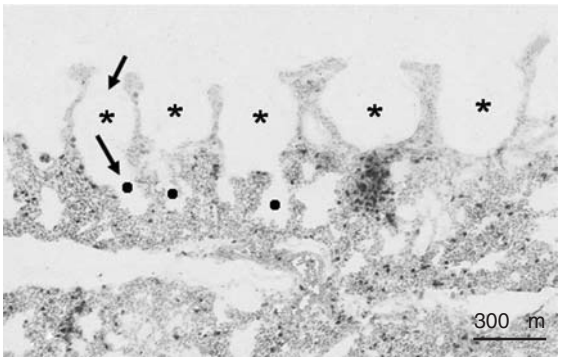

FIGURE 14.5 Section of a parabronchus. The air flow from the atrium (∗) through the infundibulum (•) into the gas exchange area is marked by an arrow. Immunohistological staining with an anti-CD4 monoclonal antibody (CT4).

a basal membrane and the blood vessel endothelium and is approximately 60% thinner than that of mammals. This provides a highly efficient gas exchange system but may also predispose the avian lung to injury from environmental irritants and pathogens (Maina, 2002).

While the air sacs are essential for ventilation of the lung, they do not participate in gas exchange. In most avian species nine air sacs are found, grouped into cranial and caudal air sacs (Fig. 14.3). They are poorly vascularized and their thin walls are covered by a squamous or cuboidal epithelium with few ciliated cells (McLelland, 1989; Crespo *et al*., 1998).

THE PARAOCULAR LYMPHOID TISSUE

The HG represents the best characterized part of the head-associated lymphoid tissue in the chicken. A detailed description of its anatomical structure can be found in Chapter 2. Briefly, the glandular lymphoid tissue is organized in two histologically distinct compartments (Olah *et al*., 1996), in which the lymphoid structure in the head of the gland with its follicle-associated epithelium (FAE), high endothelial venules (HEV) and germinal centres (GC) represent a BALT-like secondary lymphoid organ; while the plasma-cell-filled body resembles a tertiary lymphoid structure, maintaining classical immunosurveillance.

B cells in the HG are bursa dependent. This has been demonstrated in several experiments involving bursectomy, which markedly reduces the number of plasma cells in the gland (Kowalski *et al*., 1978) as well as the lachrymal immunoglobulin (Ig) concentration (Baba *et al*., 1990). Interestingly, effects of bursectomy seem to depend on the method used. While surgical bursectomy of the day-old neonate had hardly any influence on Ig production of any isotype, chemical bursectomy using cyclophosphamide slightly influenced IgM concentrations, significantly reduced the amounts of IgG and almost completely abrogated IgA secretion (Baba *et al*., 1990). Bursa dependency of HG plasma cell numbers is also supported by studies in which the infection with infectious bursal disease virus significantly reduced plasma cell numbers in the HG (Dohms *et al*., 1981). From these results, it was concluded that bursa-derived B cells migrate to the HG before hatching and that IgM could be produced by B cells of non-bursal origin (Wolters *et al*., 1977; Kowalski *et al*., 1978; Granfors *et al*., 1982; Jalkanen *et al*., 1983).

The widely-accepted concept for B cell differentiation in human and mouse implies that during a GC reaction some activated B cells differentiate first into plasmablasts and then into plasma cells. Both plasmablasts and plasma cells secrete high levels of Ig and are therefore referred to as antibody-secreting cells (ASC). While plasmablasts represent a highly proliferating intermediate stage of differentiation, plasma cells are non-dividing terminally differentiated

cells (Janeway, 1999; Manz *et al*., 2005). Gallego and Glick (1988) showed [^{3}H]-thymidine uptake in the HG not only for plasmablasts but also for mature plasma cells, while Scott and Savage (1996) observed basal bromodeoxyuridine (BrdU) uptake by HG plasma cells in young chicks; both indicating an unexpected proliferation capacity for these plasma cells. This proliferation rate was further increased after temporary plasma cell depletion by treatment with the protein synthesis inhibitor emetidine hydrochloride (Brandtzaeg *et al*., 1989; Olah *et al*., 1990; Scott and Savage, 1996). For a long time, it has been assumed that factors derived from the HG stroma influence plasma cell proliferation. This hypothesis has been supported by the demonstration of soluble stroma-derived factors which increase the proliferation rate of bursal cells (Scott and Savage, 1996). New insights might be obtained using the recently characterized chicken homologues of B-cell activating factor (BAFF; Schneider *et al*., 2004; Kothlow *et al*., 2007) and CD40L (Tregaskes *et al*., 2005). These tumour necrosis factor (TNF) family members are known to be important regulators of B cell development and function and therefore could be good candidates for those cytokines acting on B cell differentiation in the HG.

Mansikka *et al*. (1989) have shown that local immunization with tetanus toxoid vaccine leads to the production of high amounts of antigen-specific IgA and IgY antibodies but very low amounts of IgM (Mansikka *et al*., 1989). These findings are consistent with those of Burns (1976), who demonstrated bovine serum albumin (BSA)-specific antibodies with the IgA and IgY isotypes but not of IgM isotype. The results indicate that the HG can mount an immune response, in which plasma cells rapidly undergo isotype switching from IgM to IgY and IgA, respectively. Further support for this conclusion comes from the demonstration of rearrangement events (Mansikka *et al*., 1989). However, thus far neither gene conversion nor somatic hypermutation have been demonstrated in GC of the HG.

Gene expression profiling of the HG using microarray and quantitative PCR techniques revealed regulation of genes involved in Ig synthesis and downregulation of B cell receptor (BCR) signalling components (Koskela *et al*., 2003), but so far nothing is known about activation-induced deaminase (AID) expression in the various stages of differentiation of HG B cells, which could address the question of gene conversion.

Various antigens were used to investigate antigen-specific antibody production in the HG, including sheep red blood cells (Gallego and Glick, 1988; Montgomery and Maslin, 1989), *Brucella abortus* antigen (Montgomery and Maslin, 1989), Salmonella O antigen (Gallego *et al*., 1992), tetanus toxoid vaccine (Mansikka *et al*., 1989) and BSA (Burns, 1976). The route of antigen application seems to influence the outcome of the immune response. Regardless of whether antigen was applied by eyedrop or injection into the third eyelid, antigen-specific IgY antibodies increased both in the lachrymal fluid and serum. In contrast, IgA levels showed a slight increase in the serum in both groups but IgA levels in the tear fluid were significantly higher after injection into the nictitating membrane (Gallego *et al*., 1992). From these results, together with those from Survashe and Aitken (1977) – who observed higher serum antibody levels after eyedrop application in birds whose HG had been removed – several authors have concluded that parts of the lachrymal IgY have an extraglandular origin and are transported from serum to tears (Survashe and Aitken, 1977; Baba *et al*., 1990). The idea of a common mucosal immune system in the chicken has been addressed by Jayawardane and Spradbrow (1995), who showed that after oral application of a Newcastle disease vaccine increases in HG plasma cell numbers and haemagglutination-inhibition antibodies can be detected in the lachrymal fluid. Together, these data suggest that the lymphoid tissue of the HG can be considered to be immunocompetent and mount both T-dependent and T-independent immune responses.

Comparatively, little is known about the other part of the eye-associated lymphoid tissue, the CALT. Fix and Arp (1991) observed particle uptake by the lymphoepithelium of the CALT and several other groups have described the immunological development of the CALT, from randomly distributed lymphocyte aggregates in 1-week-old chicks to a BALT-like structure with organized GC and plasma cells from 4 weeks onwards (Fix and Arp, 1991; Maslak and Reynolds, 1995). Hence, it can be assumed that the CALT also contributes to the avian mucosal immunity.

THE NASAL-ASSOCIATED LYMPHOID TISSUE

The mucosal tissue of the nose is the first to come into contact with aerosols or micro-organisms during inhalation. While particles and micro-organisms may be removed mechanically by the mucus, invasive pathogens need to be controlled by the immune system. Lymphocytes have been found in the nasal mucosa, the lateral nasal glands and their secretory ducts. In the nasal cavity, lymphocytes are primarily present in, or below, the respiratory epithelium and are relatively infrequent in the vestibular region at the nasal entrance and in the olfactory region (Ohshima and Hiramatsu, 2000). While $CD8^+$ cells are distributed in the epithelium and the lamina propria, $CD4^+$ T cells are largely confined to organized lymphoid structures which develop in the subepithelial layer. These structures were called the NALT (Ohshima and Hiramatsu, 2000) and considered to be part of a common mucosal immune system, as defined for several mammalian species (Brandtzaeg *et al.*, 1997). The major characteristics of chicken NALT are the formation of circumscribed B cell areas occasionally displaying GC, that are covered by a cap of $CD4^+$ T cells. The surface of these lymphoid follicles is covered by a non-ciliated epithelium. Ig^+ B cells are found within the NALT structures and distributed throughout the epithelium, similar to the $CD8^+$ cells. The dominant Ig isotype in both locations is IgY, whereas IgM^+ cells are less frequent and IgA^+ cells are relatively rare (Ohshima and Hiramatsu, 2000).

Lymphoid structures, which can be found in the lateral nasal glands and in their secretory duct in the chickens and quail (Bang and Bang, 1968), display a similar lymphocyte subset distribution pattern as the vestibular epithelium of the nasal cavity (Ohshima and Hiramatsu, 2000). Little information is available regarding the response of these structures to antigenic challenge. Interestingly, birds raised under germ-free conditions develop the same lymphoid structures, with undistinguishable kinetics, as those kept in a specified pathogen-free (SPF) environment or under commercial conditions (Bang and Bang, 1968). Even though the available literature consists of a few publications, these consistently report the presence of T and B cells and a mucosa-associated lymphoid tissue (MALT) in the nasal cavity of birds.

THE CONTRIBUTION OF THE TRACHEA TO RESPIRATORY TRACT IMMUNE RESPONSES

Constitutive lymphoid tissue has not been described in the avian trachea. However, infection models with *Mycoplasma gallisepticum* have shown that the tracheal mucosa is highly responsive to infections and reacts with extensive lymphocyte infiltration followed by lymphoproliferation (Gaunson *et al.*, 2000, 2006). $CD8^+$ cells are found in clusters or lymphoid follicle-like structures within the tracheal mucosa while $CD4^+$ cells are spread randomly. Tracheal lesions characteristic for *Mycoplasma* infections predominantly consist of proliferating B cells (Gaunson *et al.*, 2006). Similar responses in the tracheal mucosa have been observed after infection with infectious bronchitis virus (IBV). Production of IBV-induced lesions is associated with massive lymphocyte infiltration in the tracheal lamina propria, the generation of numerous lymphoid follicles and the immigration of plasma cells (Kotani *et al.*, 2000a, b). Interesting findings have been provided in a study by Javed *et al.* (2005), who compared immune responses to *M. gallisepticum* infection in the trachea between vaccinated and unvaccinated chickens. While unvaccinated chickens had infiltration of large numbers of B and T cells and some plasma cells, vaccinated birds developed secondary lymphoid follicle-like aggregates with far fewer lesions. In addition, in tracheal tissue of vaccinated birds manifold more mycoplasma-specific ASC could be identified than in unvaccinated animals. This indicates that the tracheal mucosa lymphoid tissue can be induced by different infections.

THE BRONCHUS-ASSOCIATED LYMPHOID TISSUE

The avian lung exhibits both highly organized lymphoid structures and diffusely distributed lymphoid and myeloid cells. This was first described by Bienenstock *et al.* (1973), who compared the lung immune systems of birds with those of several mammals and found lymphoid nodules

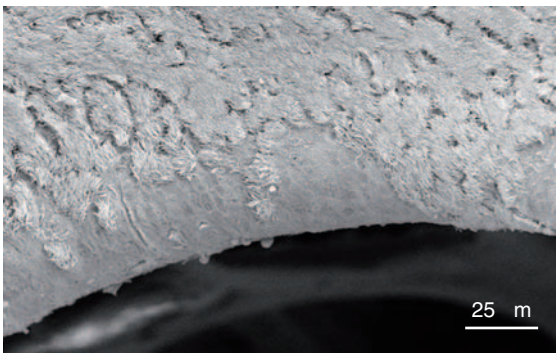

FIGURE 14.6 Ciliated and non-ciliated epithelium covering the BALT structure at the opening of the primary to a secondary bronchus (SEM preparation).

in the primary bronchus that had much similarity with Peyer's patches and other gut-associated lymphoid tissues (GALT). Hence, these structures were designated BALT. It was observed that BALT structures were constitutively present and far more frequent in the chicken than in any other species. However, this work was not followed up until growing interest in poultry respiratory diseases led to more detailed studies utilizing newly developed chicken leukocyte-specific monoclonal antibodies (mAb; Van Alstine and Arp, 1988; Jeurissen *et al.*, 1994).

BALT structures develop at the junctions of the primary bronchus and the caudal secondary bronchi (Fig. 14.2; Van Alstine and Arp, 1988; Fagerland and Arp, 1993b) as well as at the ostia of the air sacs (Myers and Arp, 1987). While they are completely absent from the junctions with the cranial secondary bronchi in chickens (Fagerland and Arp, 1993b), they are found regularly in the cranial part of the primary bronchus of turkeys (Fagerland and Arp, 1990). At hatching no, or very few, lymphocytes are present at these locations (Fagerland and Arp, 1990, 1993a, b) and organized BALT nodules are not observed prior to the third to fourth week after hatching (Fagerland and Arp, 1990, 1993a). A fully developed BALT is found in birds that are 6 weeks of age or older. It surrounds the entire openings of the secondary bronchi (Fagerland and Arp, 1990) and displays distinct B and T cell areas similar to other MALT structures (Jeurissen *et al.*, 1994).

The mature BALT is covered by a distinct layer of epithelial cells called the FAE that harbours numerous lymphocytes (Bienenstock and Befus, 1984; Fagerland and Arp, 1993a). It is made up of ciliated and non-ciliated cells in variable numbers depending on the developmental stage and is devoid of goblet cells (Fig. 14.6). Some FAE cells have irregular microvilli on their luminal surface and are in close contact with lymphocytes and myeloid cells suggesting that they may be homologous to the M cells of Peyer's patches. However, particle uptake by these cells could not be demonstrated and it remains to be seen how lymphocytes in the chicken BALT encounter antigen (Fagerland and Arp, 1993b). In the BALT nodules of 6–8-week-old chickens large B cell follicles are found (Fig. 14.7b) which are primarily made up of IgM^+ cells while IgY^+ and IgA^+ B cells are present in much smaller numbers (Fagerland and Arp, 1993a). Both in the chicken and in the turkey, GC are developed in most BALT nodules and are covered by a cap of $CD4^+$ T cells (Fig. 14.7a). In contrast, $CD8^+$ T cells are diffusely distributed between the lymphoid nodules and, as for $CD4^+$ T cells, are infrequent in the GC. Plasma cells are confined to the edge of the lymphoid tissue and found under the FAE (Fagerland and Arp, 1993a).

BALT development is influenced by age and by environmental stimuli (Jeurissen *et al.*, 1994). Single T lymphocytes are already present at predetermined locations in day-old chicks and turkey poults, while B cells are not evident until the second week. During the following weeks increasing numbers of $CD45^+$ cells accumulate and develop into organized structures with T cells in the centre and B cells at the periphery (Fagerland and Arp, 1993b; Jeurissen *et al.*, 1994). As discussed, this pattern changes in older birds where B cell areas are found in the centre of the lymphoid nodules, which are covered by $CD4^+$ T cells. GC formation is not observed until the end of the second week in conventionally-reared birds and takes one more week in SPF chickens (Fagerland and Arp, 1993a). GC are composed of blast-like cells, which stain positive

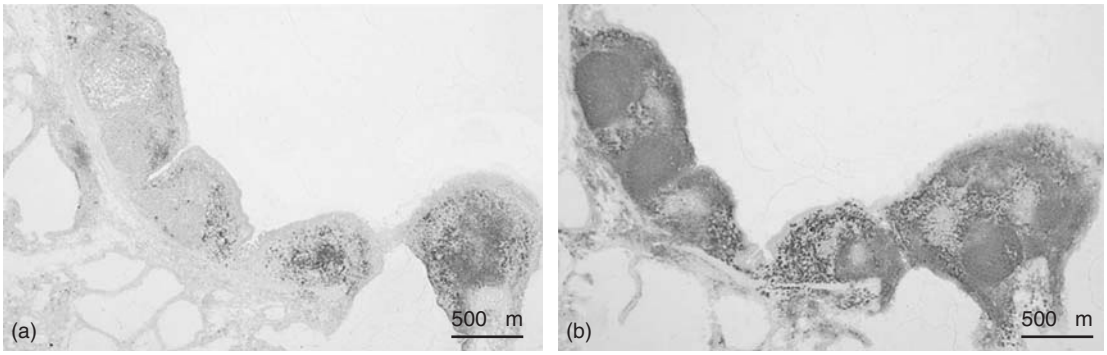

FIGURE 14.7 A fully developed BALT structure in a 12-week-old chicken. Immunohistological preparation stained with (a) an anti-CD4 (CT4) and (b) an anti-chB6 (AV20) monoclonal antibody.

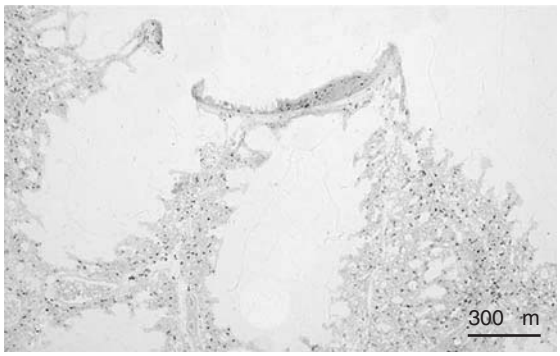

FIGURE 14.8 Diffusely distributed cells of the myeloid lineage in the lung tissue of an 8-week-old chicken stained with the monoclonal antibody KUL01.

for a pan-B cell marker (recognized by mAb HIS-C1 or CB3) and with anti-Ig antibodies, as well as IgM- or IgY-bearing cells with follicular dendritic cell morphology (Jeurissen *et al*., 1989a; Fagerland and Arp, 1993a).

If environmental stimuli are essential for BALT development is largely unknown. Early studies with germ-free chickens described lymphocytic infiltrates in the HG and the trachea but did not investigate the lung (Bang and Bang, 1968). BALT development was shown to follow a similar time course in SPF and conventional chickens, even though slight differences were observed with regards to GC development (Fagerland and Arp, 1993a). On the other hand, infections with pathogenic micro-organisms increase the number of BALT nodules significantly (Van Alstine and Arp, 1988). Whether this response is an indication of local antigen-specific T and B cell responses or due to the immigration of lymphocytes remains to be demonstrated.

THE IMMUNE SYSTEM IN THE GAS EXCHANGE AREA

Surprisingly, little is known about the structure and functional relevance of the immune system in the lung parenchyma (Reese *et al*., 2006). Immunohistological staining for a pan-leukocyte marker (using mAb HIS-C7) revealed the presence of diffusely distributed leukocytes in the interstitial tissue of the lung (Jeurissen *et al*., 1989a; Fig. 14.5). The majority of these cells were identified as belonging to the monocyte/macrophage (Fig. 14.8) and dendritic cell type because of their expression of major histocompatibility complex (MHC) class II molecules and their reactivity with the myeloid cell mAb CVI-ChNL-68.1 and -74.2 (Jeurissen *et al*., 1989b). Thus, the lung parenchyma is well equipped with resident phagocytes and antigen presenting cells (APC) which are found in the interstitial tissue and in the parabronchial wall as early as 5 days after

hatching. Lymphocytes are also widely distributed in the tissue. IgM-expressing cells appear in 1-week-old chicks, while IgA^+ and IgY^+ cells are not found at that time. At later stages $chB6^+$ (sometimes referred to as $Bu1^+$) B cells and $CD3^+$ T cells are frequently observed. Most of these T cells show the classical cytotoxic T lymphocyte phenotype with the expression of CD8 and α/βTCR molecules. $CD4^+$ cells and γ/δT cells are less frequent (Jeurissen *et al.*, 1994). While these reports clearly document the presence of an extensive system of immune cells throughout the gas exchange tissue no studies have been published addressing its function and responsiveness to antigen challenge. In this context, it is interesting to note that inhaled particles smaller than 0.1 μm in diameter reach the deep lung tissue and are taken up by the epithelial cells of the atria and infundibula and by interstitial macrophages (Stearns *et al.*, 1987). Thus, the APC and lymphocytes of the parenchyma come into contact with inhaled antigens and may initially mount a local response but subsequently help to induce a systemic antigen-specific immune response. Support for this hypothesis comes from the frequent observation of lymphoid follicles with distinct T and B cell areas in the interstitium between the parabronchi.

THE PHAGOCYTIC SYSTEM OF THE RESPIRATORY TRACT

In the alveolar lung of mammals, macrophages are not only found in the tissue, but also are present on the epithelial lining of the alveoli as a constitutively present cell population. They provide a first line of defence at the gas–blood barrier (Brain, 1985; Bowden, 1987) and play a role in immunopathological events. Alveolar macrophages can easily be obtained for functional studies using lung lavage. Application of this method to chickens or turkey yields comparatively few cells in the lavage fluid, as reported by several groups (Ficken *et al.*, 1986; Toth and Siegel, 1986; Maina and Cowley, 1998; Nganpiep and Maina, 2002). Phagocytic cells were estimated to be 20-fold less frequent in avian lavage samples than in those obtained from mice or rats (Toth *et al.*, 1987), or even considered to be entirely absent (Qureshi *et al.*, 1994; Klika *et al.*, 1996). This discrepancy can be explained by differences in the lavage procedure, the immune status of the chicks and the strain used for the study (Toth, 2000). In addition, phagocytic cells not only may be derived from the lung but also in variable amounts from the air sacs, as reported for both the chickens and ducks (Nganpiep and Maina, 2002). Phagocytic cells obtained by lavage have been either named "avian respiratory macrophages or phagocytes" (ARP; Fulton *et al.*, 1990; Toth, 2000) or "free avian respiratory macrophages" (FARM; Toth and Siegel, 1986; Maina, 2002).

The origin and the distribution of FARM in the avian lung have been addressed using microscopic techniques. These have shown that macrophages are absent from the surface of the air capillaries but present on the epithelial lining of the atria and infundibula (Maina and Cowley, 1998; Maina, 2002). Interestingly, macrophages are also abundant in the connective tissue below this epithelium on the floor of the atria (Klika *et al.*, 1996; Maina and Cowley, 1998) and in the interatrial septa (Reese *et al.*, 2006), indicating that phagocytic cells are strategically located at the start of the gas exchange area to clear the air of inhaled particles before it reaches the thin and vulnerable air capillaries.

Leukocytes are present on the surface of the air sacs. Granulocytes make up the majority of these cells followed by macrophages, while lymphocytes are relatively rare (Crespo *et al.*, 1998). As in the lung parenchyma, mononuclear phagocytes can be detected in the connective tissue of the air sacs (Klika *et al.*, 1996).

This distribution pattern of macrophages in the avian lung suggests that, even under non-inflammatory conditions, phagocytes can migrate from the connective tissue to the surface of the gas exchange area (Ficken *et al.*, 1986; Toth and Siegel, 1986), even though some authors have argued against this happening (Klika *et al.*, 1996). Repeated lung lavages lead to an increase in macrophage numbers in the lavage fluid, indicating that macrophages can transmigrate into the air space either from the connective tissue or from the vascular system (Nganpiep and Maina, 2002).

Under inflammatory conditions heterophils and macrophages are rapidly attracted to the respiratory surface. A variety of stimuli have been used to characterize this process. Inoculation of incomplete Freund's adjuvant into the abdominal air sac induces large numbers of activated FARM into the lung and air sacs. These cells are highly adhesive onto glass surfaces, actively phagocytic and cytotoxic for *Esherichia coli* as analysed by *in vitro* assays (Ficken *et al*., 1986). Likewise, phagocytic cells from chickens elicited by intratracheal introduction of live bacteria show increased phagocytic activity compared with control cells (Toth *et al*., 1988a, b). This response was already evident within 2 h of stimulation and showed peak activity about 6 h later (Ochs *et al*., 1988). Furthermore, injection of *Pasteurella multocida* into the caudal thoracic air sac led to the rapid influx of large numbers of highly phagocytic heterophils with a minor population of macrophages (Ficken and Barnes, 1989). The intratracheal introduction of heat-killed *Propionibacterium acnes* (Toth *et al*., 1987) or *Salmonella typhimurium* (Toth *et al*., 1992) also rapidly elicited FARM. On the contrary, *P. acnes*, *E. coli* lipopolysaccharide (LPS), *Saccharomyces cerevisiae* glucan or incomplete Freund's adjuvant given intravenously induced only a weak, or no, macrophages efflux from the lung (Toth *et al*., 1987). This was also demonstrated using a *P. multocida* vaccine which, after intratracheal but not after oral application, elicited phagocytic cells in the lung (Hassanin *et al*., 1995).

HANDLING OF PARTICLES IN THE RESPIRATORY TRACT

In conventional poultry production, birds are exposed to high loads of aerosolized particles. Removal of these particles takes place in all parts of the respiratory tract with larger particles controlled in the upper respiratory tract and smaller ones reaching the more distal respiratory tissue. Several independent mechanisms complement each other to prevent tissue damage and control infections. The site of deposition of inhaled particles primarily depends on particle size. As shown by Hayter and Besch (1974), larger particles with diameters between 3.7 and 7 μm are trapped and removed in the nasal cavities and trachea. Smaller particles (~1.1 μm) are transported into the lung and the cranial air sacs while particles smaller than 0.1 μm pass through the lung and are deposited in the caudal air sacs. Clearance mechanisms in the upper respiratory tract and in the trachea primarily rely on the particles being trapped in mucus, its oral mucociliary transport and expectoration or swallowing of the trapped material. A ciliated epithelium is present in the trachea, the primary bronchus and the proximal parts of the secondary bronchi (McLelland and MacFarlane, 1986) while ciliated cells are rare in the air sacs (McLelland, 1989; Crespo *et al*., 1998). Thus, particles transported beyond the ciliated mucosa must be removed by cellular mechanisms. Using aerosolized inert iron particles with a diameter of 0.18 μm, Stearns *et al*. (1987) showed that particles were phagocytosed by FARM in the gas exchange area and endocytosed by epithelial cells lining the atria and the proximal parts of the infundibula. Iron particles were also found inside those tissue macrophages underneath the atrial epithelium. This observation with the duck was subsequently confirmed by others for the chicken and pigeon and demonstrated using different foreign particular material, as well as red blood cells (Maina and Cowley, 1998; Nganpiep and Maina, 2002). It appears that birds have developed several lines of defence to protect the thin and vulnerable gas exchange tissue if particles that are too small to be removed in the upper respiratory tract are transported into the deeper lung. Obviously, FARM and the epithelial cells of the atria and infundibula are the first to encounter and remove particles from the luminal surface of the avian lung (Maina and Cowley, 1998; Maina, 2002). In the tissue, subepithelial phagocytes, interstitial macrophages (Jeurissen *et al*., 1994; Lorz and Lopez, 1997; Scheuermann *et al*., 1997) and a poorly defined population of resident pulmonary intravascular macrophages (Maina and Cowley, 1998) remove foreign particles which have crossed the epithelial barrier. The subsequent fate of this material is still not known. Theoretically, it could be transported from the lung to secondary lymphoid organs, as demonstrated using mammals. Alternatively, birds may present processed antigen locally to mount an antigen-specific immune responses either in the constitutively

present BALT or in organized lymphoid nodules which are inducible in many avian tissues by antigen inoculation. Such lymphoid aggregates have been observed in the avian lung parenchyma but their function is still unclear.

The handling of inhaled particles in the air sacs is even less well understood. Although a ciliated epithelium was described in the most proximal parts of the air sacs (Crespo *et al*., 1998), transport of the particles back into the bronchi has not been demonstrated experimentally. Therefore, phagocytic cells of myeloid origin are primarily responsible for particle clearance, even though this process is significantly slower than in the lung tissue (Mensah and Brain, 1982; Ficken *et al*., 1986).

THE SECRETORY IgA SYSTEM IN THE RESPIRATORY TRACT

Antigen-specific protection of mucosae in the upper airway is achieved mainly using the humoral immune system (Brandtzaeg, 1995; Phalipon *et al*., 2002) through the production and secretion of polymeric IgA and IgM (Brandtzaeg *et al*., 1997). These immunoglobulins are transported through the epithelial cells by a basolaterally expressed receptor protein, the poly-Ig receptor (Snoeck *et al*., 2006). The avian homologue of this receptor has been recently cloned (Wieland *et al*., 2004), but its expression pattern in epithelial cells of the respiratory tract is so far not known.

Secretory Ig perform immune exclusion by inhibiting the uptake of soluble antigens and by blocking adhesion and invasion of epithelia by micro-organisms (Avakian and Ley, 1993; Snoeck *et al*., 2006). As discussed above, numerous B cells and ASC are present in the head-associated lymphoid tissues and throughout the respiratory tract of avian species, and Ig have been detected in the tears and lachrymal fluid (Mansikka *et al*., 1989; Jayawardane and Spradbrow, 1995), nasal (Takada and Kida, 1996), tracheal (Jayawardane and Spradbrow, 1995; Gaunson *et al*., 2000) and lung washings (Avakian and Ley, 1993; Tizard, 2002; Javed *et al*., 2005).

Secreted Ig in the respiratory tract are produced by bursa-derived cells (Lam and Lin, 1984), primarily of the IgA isotype, although IgY and IgM antibodies are also found (Russell, 1993). Using a haemolytic plaque assay, Lawrence *et al*. (1979) demonstrated that the respiratory tissue is relatively enriched in IgA-secreting cells which could be the main source of mucosal IgA. The mucosal IgA response is T cell dependent, since *in vivo* depletion of α/β T cells almost completely abolishes IgA production (Cihak *et al*., 1991). The protective role of secretory (s)IgA has been investigated in several infection and vaccination studies with economically important respiratory pathogens. Intranasal or eyedrop vaccination with Newcastle disease virus (NDV) induces antigen-specific IgA antibodies which can be detected in tears and tracheal washings (Jayawardane and Spradbrow, 1995; Russell and Ezeifeka, 1995; Ganapathy *et al*., 2005). Application of a live virus vaccine does not seem to be essential since inactivated NDV given by the intranasal route elicits a protective sIgA and sIgM response, which can be further enhanced by addition of the mucosal adjuvant, cholera toxin B subunit (Takada and Kida, 1996). Comparable results were obtained in *M. gallisepticum*-infected birds which developed antigen-specific IgA, IgM and IgY responses in washings of the upper and lower respiratory tract (Yagihashi and Tajima, 1986). Functional *in vitro* assays demonstrated that these antibodies were protective but that protection did not correlate with the IgA titres in the washing, indicating that secreted IgM and IgY are also of relevance in mucosal defence (Avakian and Ley, 1993). Intratracheal infection with *M. gallisepticum* induces the accumulation of IgY^+ and IgA^+ B cells and plasma cells in the lamina propria. In contrast, vaccination prior to infection induced the formation of lymphoid follicles and of strongly elevated numbers of antigen-specific ASC as measured by an enzyme-linked immunospot (ELISPOT) technique. It also leads to a significantly higher proportion of *M. gallisepticum*-specific IgA and IgY antibodies in tracheal washings (Javed *et al*., 2005). While IgA is most probably produced locally and secreted as a polymeric antibody, IgY antibodies may be locally secreted or transudated from the serum as suggested by several studies (Toro *et al*., 1993; Suresh and Arp, 1995; Javed *et al*., 2005). Finally, induction of

antigen-specific IgA and IgY antibodies in tear fluid and lower respiratory tract lavage samples have been demonstrated in IBV-vaccinated birds (Toro and Fernandez, 1994; Thompson *et al*., 1997) and was induced equally well by antigen delivery through ocular instillation, spray or drinking water application (Toro *et al*., 1997).

FUTURE DIRECTIONS

Most of our knowledge about the immune system of the respiratory tract is based on morphological studies. Functional studies have largely been limited to the demonstration of antigen-specific antibody responses. Advances in avian genomics and the availability of new technologies such as the quantitative PCR, microarray techniques and transgene technology (see Chapter 11) should enable more detailed investigations about the function of this mucosal defence system.

Innate protective factors are known to be important in the defence of the mammalian respiratory tract. Recently, 13 different chicken β-defensins, designated gallinacins, have been identified by screening of expressed sequence tags (EST) and genome sequence databases (see Chapter 7). Gene expression in the trachea and in the lung was restricted to Gal-1, Gal-2, Gal-3, Gal-5 and Gal-7 (Xiao *et al*., 2004) and tracheal expression of Gal-3 was significantly upregulated in response to *Haemophilus paragallinarum* infection (Zhao *et al*., 2001). Using a similar approach, the family of chicken collectins has been identified and their expression, together with the expression of surfactant protein A (SP-A), has been demonstrated in the lung and in the tracheal tissue (Zeng *et al*., 1998; Hogenkamp *et al*., 2006). Progress in the identification of these antimicrobial peptides will help us to better understand the host response to pathogens, to identify host resistance factors and develop new therapeutic or preventative strategies for poultry farming, based on natural antimicrobial components (Lazarev *et al*., 2004).

Gene expression analysis by microarray technology is now increasingly used to study host–pathogen interaction in more detail and first results have been published for IBV (Dar *et al*., 2005) and influenza infections in the lung (Degen *et al*., 2006). In addition, cytokine expression studies on isolated lung lymphocytes indicated an important role for $CD8^+$ T-cell-derived interferon-γ in the protective immunity to influenza virus (Seo *et al*., 2002). As discussed, the immune system of the avian lung is compartmentalized into organized BALT structures and diffusely distributed lymphocytes and myeloid cells in the gas exchange tissue. These structures will most likely respond to infections differently thus necessitating focused tissue sampling for expression and functional studies in order to avoid misinterpretation of collected data. Laser dissection microscope guided probe sampling should be considered as one practicable way to achieve optimal results.

Post-hatch immunization of poultry is frequently achieved by aerosol, spray or eyedrop vaccination and activates both the local and systemic adaptive immune systems. Improvement of vaccines by rational design requires a comprehensive understanding of the way in which antigen is taken up, processed and presented to the head-associated respiratory immune system of birds, a process that is not understood. Therefore, future research will have to focus on the characterization of the phenotype and function of professional APC in the respiratory tract and on methods which will allow direct targeting of antigen to APC and their activation by new adjuvants.

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15 THE AVIAN REPRODUCTIVE IMMUNE SYSTEM

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INTRODUCTION
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INTRODUCTION

The avian reproductive tract differs greatly from that of mammals in both its structure and function. Infection of the reproductive tract and particularly infection of developing eggs has consequences in the vertical transmission of disease to progeny and, in the case of *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis), infection of table eggs for human consumption is a major public health issue. The immune response in the reproductive tract to these infections is poorly understood. Indeed, the structure and role of the immune system in the reproductive tract is arguably one of the least appreciated areas of avian immunology. In this chapter, we review what is known of the immune system in the reproductive tract, its development associated with sexual maturity and its role during infection.

THE STRUCTURE AND FUNCTION OF THE AVIAN REPRODUCTIVE TRACT

The reproductive tract of female chickens consists of a functional left ovary and oviduct with vestigial organs on the right side (King and McLelland, 1984). The ovary reaches maturity from around 17 to 25 weeks of age, depending on the breed or strain of chicken (Gilbert, 1971). The immature ovary consists of a central medulla surrounded by a cortex which contains the

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developing oocytes and is surrounded by cuboidal epithelial cells. As the bird matures, a distinct hierarchical follicular structure develops with thousands of developing follicles present within the ovary. Towards sexual maturity a number of follicles begin to mature rapidly, growing up to 40 mm in diameter. The mature follicles consist of a number of distinct layers surrounding the oocyte or yolk. Immediately surrounding the yolk is the vitelline membrane. This is surrounded by a perivitelline layer and the ovarian granulosa, which in turn is surrounded by a basement membrane, layers of theca and connective tissue and finally an epithelial layer (Hodges, 1974).

Following ovulation, the mature oocyte or yolk is deposited into the oviduct (Aitken, 1971). The oviduct is a convoluted tubular structure that stretches from the ovary to the cloaca. In mature hens, it is around 60–80 cm long when fully extended. During passage through the oviduct the albumen, membranes and shell of the egg are deposited around the yolk. The first section of the oviduct is the infundibulum, which receives the developing egg from the ovary. This is a funnel-like structure which has considerable secretory activity with goblet cells secreting mucus and ciliated cells facilitating movement down the oviduct. The developing egg passes into the magnum, the longest part of the oviduct which secretes albumen around the yolk. Like the infundibulum the magnum contains numerous ciliated and secretory cells (Hodges, 1974). The mucosal structure of the magnum is highly folded with underlying pyramidal proprial gland cells that secrete much of the albumen protein. The egg then passes into the isthmus which produces the shell membranes. Following the deposition of the membrane the egg passes into the uterus. It is retained within a pouch-like structure, in which calcification of the egg to form the shell occurs over a period of around 20 h. The shelled egg then passes into the vagina and eventually layed through the cloaca. The vagina also acts as a depository for spermatozoites after mating, these are released gradually to fertilize eggs in the ovary.

The male reproductive tract is, perhaps, less of an issue in terms of infectious disease and vertical transmission of infection. It consists of two internalized testes. In the chicken these are located near the kidneys and are 5–6 cm in length in mature birds. As in mammals spermatozoa develop within the testes. Unlike mammals there are no accessory genital organs and seminal fluid is also produced by the testes. Semen is secreted via ducts to the epididymis and to the ductus deferens which connects to the phallus. Birds may have a protruding phallus, as in ratites or many waterfowl, or non-protruding as in galliforms and most passerines.

STRUCTURE AND DEVELOPMENT OF THE REPRODUCTIVE TRACT-ASSOCIATED IMMUNE SYSTEM IN THE CHICKEN

Organization of Lymphocytes in the Reproductive Tract

There are a number of descriptions of lymphocyte organization within the ovaries and oviduct. Histology and immunohistology have been largely utilized to determine these, though as yet there are very few functional studies. Early histological examination indicated the presence of scattered lymphocytes and lymphocyte aggregates in the oviduct (Biswal, 1954). The distribution of immunoglobulin (Ig)-secreting cells within the reproductive tract was described in the 1970s, including the distribution of cells secreting IgA (Lebacq-Verheyden *et al*., 1974; Lawrence *et al*., 1979). These studies indicated the presence of large numbers of IgA-secreting cells below the oviduct epithelium. Localization of B lymphocytes and their associated Ig subclasses was described by Kimijima *et al.* (1990). This indicated the presence of IgY^+ B lymphocytes throughout the oviduct associated with both epithelial and glandular tissue, with highest numbers associated with the epithelium of the infundibulum, the glandular regions of the magnum and the uterus. IgM^+ and IgA^+ B lymphocytes are found more frequently in glandular tissue of the magnum, though scattered cells are found throughout most areas of the oviduct (Kimijima *et al*., 1990). Withanage *et al.* (1997) investigated the distribution of B lymphocytes in the reproductive tract of mature hens and indicated that scattered cells are present throughout the oviduct, although

they are found infrequently in the ovary and those present are IgM^+ and IgA^+ cells rather than IgY^+ B lymphocytes. IgA^+ B cells are found primarily under the epithelium, IgY^+ B cells with tubular glands and IgM^+ B lymphocytes with the magnum and isthmus (Withanage *et al.*, 1997). *In situ* hybridization of IgY γ-chain mRNA showed the presence of IgY^+ B lymphocytes throughout the oviduct with greater numbers of γ-chain expressing cells associated with the stroma than the mucosa (Zheng *et al.*, 2000). The oviduct is also considered to be the main site of maternal transfer of antibody to the egg, with IgM and IgA present in the albumen, and IgY in the yolk.

T lymphocytes are present in both the ovary and the oviduct (Withanage *et al.*, 1997). T lymphocytes are also associated with the loose lymphoid aggregates or nodes present in the oviduct. $CD4^+$ lymphocytes are found most frequently in the lamina propria and are most numerous in the vagina. $CD8^+$ cells are frequently associated with the epithelium and appear to form a major proportion of intraepithelial lymphocytes (IEL). $CD8^+$ T lymphocytes are most numerous in the vagina and the infundibulum of the oviduct. Unlike B lymphocytes, both $CD4^+$ and $CD8^+$ T lymphocytes are found in significant numbers, associated with the ovaries (Withanage *et al.*, 1997). Large granular lymphocytes have also been described in the oviduct (Khan and Hashimoto, 2001). These are associated with glandular cells and the epithelium and are most numerous in the magnum and vagina.

Distribution of Macrophages and Other Cells

Immunocytochemical staining has indicated the presence of macrophages or macrophage-like cells in the ovarian follicles of the chicken (Barua *et al.*, 1998a, 2001; Barua and Yoshimura, 1999a, b). Major histocompatibility complex (MHC) class II positive cells have been found in the thecal layer of preovulatory follicles and in the theca and granulosa of postovulatory follicles (Barua *et al.*, 2001). Macrophages are also present throughout the length of the oviduct, most frequently in the infundibulum and vagina (Barua *et al.*, 1998a; Withanage *et al.*, 1998; Khan and Hashimoto, 2001). The distribution of heterophils within the reproductive tract is less clear, although early reports indicate the presence of eosinophilic cells in the ovarian medulla (Hodges, 1974).

EFFECTS OF AGE AND STEROIDAL HORMONES ON IMMUNE CELL POPULATIONS IN THE REPRODUCTIVE TRACT

The onset of sexual maturity has pronounced effects on the reproductive tract-associated immune system. Attempts have been made to induce these changes through the administration of sex steroids, and in particular the oestrogen diethylstilboestrol (DES) (Barua *et al.*, 1998a; Khan and Hashimoto, 2001). Although such an approach has some value, it can be argued that the levels administered have little significance to real physiological levels and its use is evidently detrimental to the health of experimental hens. However, such studies suggest that raised levels of oestrogens lead to increases in both T cells and Ig-secreting cells (Barua *et al.*, 1998a, b). Large granular lymphocytes are first detected in the oviduct at 9 weeks of age and increase to a peak between 21 and 24 weeks of age following sexual maturity (Khan and Hashimoto, 2001). Administration of DES also increases their numbers in the oviduct.

Macrophages were also increased in the ovary of young laying hens compared with immature or older birds (Barua *et al.*, 1998c). Administration of DES, but not progesterone, led to a significant increase in macrophage numbers. Similarly, macrophage numbers increase to a peak in the oviduct following the onset of sexual maturity (Zheng and Yoshimura, 1999). Any functional change associated with the increase in immune cell numbers in the reproductive tract of hens following the onset of sexual maturity is not yet known. It has been proposed that these changes are to prevent infection or to "clean" the oviduct at the commencement of egg laying, but this is pure speculation in the absence of any functional studies.

In addition to a role in immunity to infection, ovarian macrophages and/or MHC class II^+ positive cells are believed to play a role in development of ovarian follicles and particularly in the regression of postovulatory follicles (Barua *et al.*, 1998a, b, c, 2001).

THE REPRODUCTIVE TRACT IMMUNE SYSTEM IN INFECTION

Bacterial Infections of the Reproductive Tract

A number of bacteria may infect the reproductive tract of chickens, including some strains of *Escherichia coli* that lead to egg peritonitis and salpingitis (Jordan *et al.*, 2005). However, the most studied and best described bacterial infection of the reproductive tract is *S. enterica*. Two serovars have a particular affinity with reproductive tract infection. These are *S. enterica* serovar Enteritidis, the serovar most associated with human food-borne salmonellosis and serovar Pullorum, the cause of the systemic pullorum disease. Both serovars are frequently vertically transmitted by egg infection.

Much work on avian salmonellosis has concentrated on egg infection by *S.* Enteritidis, and a number of bacterial virulence factors have been implicated in this process (Guard-Petter, 2001; De Buck *et al.*, 2004). The bacterium infects both developing and newly formed eggs in the ovary and oviduct (Keller *et al.*, 1995; De Buck *et al.*, 2004). The rate of egg infection with *S.* Enteritidis is low, both experimentally and in production (Keller *et al.*, 1995; De Buck *et al.*, 2004). The exact nature of the infection is also poorly understood, as it is not completely clear whether *S.* Enteritidis infects at a young age, persists and infects eggs, or infection is associated with the laying period. Although some studies suggest that birds infected at a young age develop a carrier state that leads to egg infection (De Buck *et al.*, 2004), others suggest that it is almost impossible to reproduce experimentally (Berchieri *et al.*, 2001). Given the low frequency of infection, it seems likely that a carrier state could develop in a small number of animals, and be genetic in origin. Infection of the reproductive tract and transmission to eggs is considerably more frequent with *S.* Pullorum, where about half of experimentally infected hens develop a carrier state and the reproductive tract becomes infected (Wigley *et al.*, 2001). In this case, the site of infection is predominately within splenic macrophages, with reproductive tract infection becoming prevalent only at sexual maturity. The ability of *S.* Pullorum to persist within macrophages is dependent on the *Salmonella* pathogenicity island 2 (SPI2) type III secretion system, a bacterial apparatus that translocates bacterial effector proteins or toxins to the host cell. The primary function of SPI2 is to interfere with intracellular trafficking within macrophages thereby inhibiting phagolysosome fusion (Wigley *et al.*, 2002). In mammalian species, the *S.* Typhimurium SPI2 system has also been shown to modulate the immune response, in particular inducing interleukin (IL)-10-mediated regulatory responses in maintaining persistent infection (Uchiya *et al.*, 2004). It is possible that the *S.* Pullorum system plays a similar role.

The Immune Response to *Salmonella* Infection of the Reproductive Tract

The immune response to *Salmonella* infection can be divided into those responses that occur systemically during infection or vaccination and those local responses that occur within the reproductive tract. We will first deal with the systemic response. Studies with killed and live *S.* Enteritidis vaccines indicate the generation of both humoral and cellular responses (Woodward *et al.*, 2002; Okamura *et al.*, 2003, 2004; Babu *et al.*, 2004). Oral infection with wild type *S.* Enteritidis has indicated a strong humoral response is generated (Berchieri *et al.*, 2001). In common with *S.* Typhimurium infection of chickens, clearance of bacteria from systemic sites is found 2–3 weeks post-infection, a time that coincides with peak expression of interferon (IFN)-γ and antigen-specific T cell proliferation (Beal *et al.*, 2004; Withanage *et al.*, 2005). Recent unpublished studies suggest that there is a peak of IFN-γ, IL-12 and IL-18 activity in the spleen at 2 weeks post-oral infection with *S.* Enteritidis (P. Wigley, P. Barrow and

P. Kaiser, unpublished observations) and, as is the case with *S.* Typhimurium, there is a peak in antigen-specific T cell proliferation at 3 weeks post-infection (Beal *et al.*, 2006). In sexually immature birds the biology of infections with *S.* Enteritidis and *S.* Typhimurium and the immune responses against both pathogens are very similar. As yet, it is not clear which elements of the response are protective against subsequent infection. Although it appears that cell-mediated responses are important, the initial successes of killed bacterins as vaccines in the control of *S.* Enteritidis in layer flocks in the United Kingdom suggest that antibodies also play an important role in protection from re-infection.

Persistent *S.* Pullorum infection also induces antigen-specific T cell responses and particularly strong humoral responses with specific IgY titres of 1 in 60 000 or greater (Wigley *et al.*, 2001, 2005b). The T cell response to mitogenic stimulation is halved in infected birds compared with controls suggesting an overall suppression of the cellular response (Wigley *et al.*, 2005b). Early in *S.* Pullorum infection the increase in expression of Th1 associated cytokines (IFN-γ, IL-18) seen in the spleen during infection with serovars Typhimurium (Beal *et al.*, 2004; Withanage *et al.*, 2005), Gallinarum (Wigley *et al.*, 2005a) and Enteritidis (P. Wigley, P. Barrow and P. Kaiser, unpublished observations) is absent, though interestingly there was a significant increase in IL-4 at the same time point (P. Wigley, P. Barrow and P. Kaiser, unpublished observations). This suggests that *S.* Pullorum generates a Th2-dominated response. This is in keeping with its infection biology in establishing persistent intracellular infection accompanied by a strong antibody response, though it appears that IFN-γ-mediated responses are generated later in the infection. We have previously hypothesized that *S.* Pullorum persists in macrophages suppressing Th1 responses, requiring the SPI2 type III secretion system for persistence, whilst the host produces a Th1-mediated response in an attempt to clear the infection. The balance of host response and pathogen immunomodulation results in the carrier state. Such a system has also been shown in mice persistently infected with *S.* Typhimurium, where administration of anti-IFN-γ antibodies pushes the equilibrium in favour of the pathogen resulting in severe systemic infection (Monack *et al.*, 2004). In the *S.* Pullorum carrier state the equilibrium is broken by the onset of sexual maturity. In hens, the onset of lay is accompanied by a dramatic decrease in antigen-specific and mitogen-stimulated T cell proliferation (Wigley *et al.*, 2005b). This has the effect of allowing replication of the *Salmonella* in the spleen and liver, along with the spread of infection to the ovaries and oviduct. Immunosuppression at the point of lay offers a clear "window of opportunity" for pathogens to infect or re-emerge from latency. Indeed, there is considerable anecdotal evidence of increased susceptibility to helminth, protozoal and viral infections at this time point as well as direct evidence of recrudescence of both *Salmonella* and infectious bronchitis virus associated with the onset of lay (Jones and Jordan, 1972). The mechanisms by which *Salmonella* is transported to the reproductive tract from the spleen are unclear, though it is conceivable that during the influx of macrophages to the reproductive tract at the onset of lay (Withanage *et al.*, 1998), cells infected with *Salmonella* are also directed to this site.

Our knowledge of the local immune response in the reproductive tract to *Salmonella* infection is even less clear. A particular limitation of the studies that have been reported is the use of intravenous or intraperitoneal challenge of old laying hens with a high inoculum of *S.* Enteritidis (Withanage *et al.*, 1998, 2003; Barua and Yoshimura, 2004). This infection model does lead to reproductive tract infection, though only as part of a transient general systemic infection with considerable pathology and very unlike the situation found in egg infection associated with younger birds. Nevertheless, these studies have shown that there is a surge of both $CD4^+$ and $CD8^+$ T cells into the ovaries and oviduct 7 days after infection, peaking at 10 days post-infection, whereas B lymphocytes peak at 14 days post-infection (Withanage *et al.*, 1998; Withanage *et al.*, 2003). Both return to pre-infection levels by 21 days post-infection. In contrast, macrophage numbers decrease initially, followed by an increase. Other recent studies suggest that the influx of T lymphocytes to the ovaries is very rapid, occurring within 12–24 h of infection (Barua and Yoshimura, 2004). *Salmonella*-specific IgY and IgM antibodies are also secreted into the oviduct following infection, determined by enzyme-linked immunosorbent assay (ELISA) of oviductal washings, with a peak at 14 days post-infection although, perhaps

unexpectedly, there was only a transient change in levels of IgA (Withanage *et al*., 1999). In these studies, the increases in both antibodies and lymphocyte numbers coincided with bacterial clearance. Due to the nature of the experimental model, it is not clear whether this is a reflection of a specific reproductive tract associated response or merely an extension of the systemic response to *Salmonella*. Antibodies have also been detected in serum and secreted into the reproductive tract in vaccinated and unvaccinated chickens challenged intravaginally (Miyamoto *et al*., 1999).

Little is known about the nature of infection and immunity in the male reproductive tract. While it is clear that *S*. Pullorum does not infect the male reproductive tract in the same way as in hens (Wigley *et al*., 2005b), the behaviour of *S*. Enteritidis is less clear. There are two reports suggesting that *S*. Enteritidis may be transmitted sexually *via* semen and that this route enhances the ability of *S*. Enteritidis to persist in the female reproductive tract (Reiber and Conner, 1995; Reiber *et al*., 1995).

Viral Infections of the Reproductive Tract

Thus far, no viruses have been identified that exclusively replicate in the reproductive tract of birds, although many viruses causing systemic infections may also replicate in the female and male reproductive tracts during the period of active viral replication. Transient transmission of the virus through the embryonated egg may also occur during the viral replication period. Normally, the development of general antiviral immune responses results in the elimination of the agent from the reproductive organs, so these infections will not be discussed in this section. However, in some instances virus may become established in the reproductive organs as a latent and/or persistent infection even in the presence of virus-neutralizing (VN) antibodies.

The following viruses are of particular interest for the establishment of latent and/or persistent infection in the reproductive tract: avian leukosis virus (ALV), reticuloendotheliosis virus (REV), chicken infectious anaemia virus (CIAV) and two genera of adenovirus (Aviadenovirus and Atadenovirus). The mechanism for the establishment of persistent infection is reasonably well understood for ALV and is probably similar for REV (Fadly and Payne, 2003; Witter and Fadly, 2003). Congenital infection with ALV or REV results in the development of immunological tolerance to these pathogens. These birds become persistently viraemic and are unable to produce VN antibodies (referred to as V^+A^-) and unable to clear the virus from the reproductive tract. In V^+A^- chickens, virus is transmitted from the albumen-secreting glands in the oviduct to the developing egg. Infection at hatching from congenitally infected chickens results mostly in V^-A^+ chickens. Some of these birds may become intermittent shedders of virus into the eggs. The reasons why the VN antibodies are unable to completely clear the infection in the uterus is not known.

Avian adenoviruses, belonging to the genera Aviadenovirus and Atadenovirus are reported to establish latent infections, which may result in subsequent reactivation and vertical transmission. Fadly *et al*. (1980) reported that aviadenovirus could remain latent in a specific-pathogen-free flock for at least one generation while the birds were positive for VN antibodies. The location of latent virus in the reproductive tract has not been studied. The mechanisms controlling virus replication and vertical transmission have not been elucidated but it has been suggested that steroid hormones could be involved (McFerran and Adair, 2003a). Egg drop syndrome (EDS) virus, the only member of the genus Siadenovirus, has also been associated with latency after infection *in ovo* and, perhaps, in newly hatched chickens. EDS virus replicates in several parts of the uterus after experimental infection but it is not clear if the virus remains latent in these sites or elsewhere. Reactivation mostly occurs when birds reach sexual maturity in hens lacking VN antibodies. It is not known if the absence of VN antibodies after *in ovo* infection is caused by the induction of tolerance (McFerran and Adair, 2003b). Reactivation may be under control of steroid hormones interacting with the virus and sequencing may show the presence of hormone response elements in the promoters of viral genes.

CIAV, the only member of the genus Gyrovirus of the Circoviridae, can be detected in the reproductive tract of hens in the absence or presence of VN antibodies, suggesting the presence of latent virus. The reasons for the lack of virus clearance are not understood but, based on serological data, it is unlikely to be caused by the induction of tolerance. Latent virus or viral DNA can be transmitted to the embryo with little or no consequences for the embryo and this latent transmission may occur over several generations (Miller *et al*., 2003). In contrast, vertical virus transmission during active infection in the hen results in clinical disease in young chickens (Miller and Schat, 2004). Viral reactivation is likely to be under control of steroid hormones such as oestrogen interacting with hormone response elements, which have been demonstrated in the promoter region of CIAV (Miller *et al*., 2005). Activation is also controlled by strong repressor elements in this region (M.M. Miller and K.A. Schat, unpublished observations). The only evidence for the presence of latent infection is the occurrence of seroconversion after sexual maturity, which can occur in a few birds or up to 100% of the flock before the end of the first laying cycle.

The immune responses in the male reproductive system to viral infection are less well understood than the responses in the female reproductive tract. Transmission through semen has been described for these viruses but their importance for virus spread is not known.

WHAT WE NEED TO KNOW – DIRECTIONS FOR FUTURE RESEARCH?

Arguably, the immune system is one of the "black boxes" of the reproductive tract with little known about its function and knowledge of its response to infection has been restricted to a few descriptive studies. There is an enormous need to improve our understanding of both basic and applied immunology of the reproductive tract. The questions below begin to address some key areas.

What are the Functions and Phenotypes of the Cells in the Reproductive Tract?

At present we know virtually nothing of the function, or phenotype, of the cells associated with the reproductive tract other than relative numbers of $CD4^+$ and $CD8^+$ T lymphocytes (Withanage *et al*., 1998, 2003). We know nothing of the distribution of avian Toll-like receptors and other receptors associated with the innate immune system in these tissues (see Chapter 7). We know nothing of the cytokines and chemokines expressed in tissues or by individual cells, either constitutively or in response to infection; something that has been addressed in many other body systems of the chicken. For example, would an intravaginal challenge with *Salmonella* or even heat-killed bacteria or lipopolysaccharide induce a pro-inflammatory cytokine and chemokine responses, or are these responses tightly controlled by regulatory cytokines similar to the control of immune responses in the gastrointestinal tract of more mature birds (Withanage *et al*., 2005)? While expression of receptors and messengers in tissues and to some extent their distribution can be addressed by quantitative reverse transcriptase PCR, *in situ* hybridization and immunohistochemistry, techniques such as laser-dissection microscopy need to be employed to address the immunobiology of individual cell types.

How Does the Reproductive Tract Integrate with the Rest of the Immune System?

The extent to which the reproductive tract integrates with the immune system as a whole and the extent to which it functions as a local system is not known. It is not clear whether localized infection in the reproductive tract induces substantial systemic responses, or whether systemic infections induce a substantial adaptive response in the reproductive tract, including responses induced by vaccination (discussed below). Our current lack of knowledge of the reproductive tract immune system makes it impossible to predict if a local infection of the reproductive tract induces a cytokine response that leads to a more extensive systemic response.

Is the Reproductive Tract Immune System Stimulated by Vaccination?

It is also unclear to what extent, if any, vaccination leads to responses in the reproductive tract. This is perhaps surprising, considering the extent to which vaccination is employed in the control of *Salmonella* infection of eggs, although as, discussed earlier, most studies have focused almost entirely on protection and the immune response to systemic and, to a lesser extent, gastrointestinal infection. Descriptive changes to vaccination would be fairly easy to determine through immunohistology, though functional changes would be difficult to achieve, as discussed earlier. Nevertheless, the importance of vaccination in control of *Salmonella* infection of eggs, and the emergence of new *S*. Enteritidis phage types in egg infection indicate that a better understanding of immunity is of paramount importance to the poultry industry.

What are the Changes in Cell Population Structure and Immune Function Associated with the Onset of Sexual Maturity?

A number of previous studies have shown that there are significant changes in cell numbers in the reproductive tract at the onset of sexual maturity. In general, this is an increase in numbers of lymphocytes. Although the reasons for this influx are not known, it appears that oestrogens are the mediator of this cellular influx. Although not directly associated with the reproductive tract itself, there is considerable suppression of systemic T cell responses at this time. The nature of these changes has not been fully characterized, though it has been assumed that they are a consequence of increased levels of steroid hormones. Whether the cells within the reproductive tract also have reduced functions is not known and, due to their rather scattered nature, may be difficult to demonstrate. Nevertheless the changes, both systemically and within the reproductive tract, are fascinating and an important demonstration of how stressors can impact on immune function. Loss of function has important implications for increased susceptibility to infections at point of lay, something for which there is considerable anecdotal evidence in free-range production. The potential consequences of this downregulation of an adaptive immunity (and any changes in innate immunity) and the hormonal, or physiological, changes that trigger the immunological changes need better characterization. Ways should be sought to enhance immune function around this time, possibly by modulating the immune system using recombinant cytokines or with nutritional supplementation.

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16 AVIAN IMMUNOSUPPRESSIVE DISEASES AND IMMUNE EVASION

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INTRODUCTION
IMMUNOSUPPRESSION
IMMUNOEVASION
CONCLUSIONS
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INTRODUCTION

Control of infectious diseases is essential for the production of healthy poultry flocks and this is generally achieved by extensive vaccination programmes in combination with good management practices including biosecurity measures to reduce the risk of infection. The success of vaccination programmes depends on the ability of the birds to mount a vigorous immune response after vaccination. In addition to the innate ability of a particular bird to mount an immune response to a vaccine or an infection there are numerous external factors influencing the level of protective immunity. The purpose of this chapter is to review the mechanisms, and impact of immunosuppression, which can have multiple causes, and the ability of pathogens to counteract immune responses by immunoevasion. Immunosuppression is defined as "*A state of temporary or permanent dysfunction of the immune response resulting from insults to the immune system and leading to increased susceptibility to disease*" as originally proposed by Dohms and Saif (1984) with the addition of "*and often a suboptimal antibody response*" as suggested by Lütticken (1997). Such dysfunction often results from infection of cells of the immune system, leading to their impaired function against the primary and subsequent infections, in a non-specific manner. For this chapter, we define immunoevasion as "*Pathogen-initiated responses counteracting the immune responses to the specific pathogen*". The major difference is that immunosuppression is the consequence of the overall replicative strategy of the causative agent, resulting in increased susceptibility to other pathogens but not necessarily to the causative agent, while immunoevasion is achieved by specific pathogen-encoded determinants, primarily favouring replication of the causative agent. In some instances, infections can result in immunosuppression and immunoevasion, e.g. infection with Marek's disease virus (MDV).

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IMMUNOSUPPRESSION

Introduction

Immunosuppression is a major problem for the poultry industry but actual figures indicating the scale of the problem are hard to find. Infection with pathogens and/or environmental factors, including management errors, can result in immunosuppression and interactions between the two usually exacerbate the problem. Any infection causing clinical disease may result in immunosuppression, but the focus will be on pathogen-induced immunosuppression in the absence of clinical disease. Viruses causing immunosuppression and/or clinical disease at any age include MDV, reticuloendotheliosis virus (REV), reovirus, and, although controversial, avian leukosis virus (ALV). Other pathogens, e.g. chicken infectious anaemia virus (CIAV), may cause clinical disease in young chicks but the major damage consists of subclinical infection causing immunosuppression. Infectious bursal disease virus (IBDV) also causes clinical disease in young chicks, but this acute infection results in damage to immune tissue, particularly the bursa of Fabricius and bursa-derived lymphocytes, compromising the host's ability to mount effective responses upon subsequent infection by other pathogens. Caution is needed, however, in the interpretation of the immunosuppressive effects caused by reovirus, MDV, REV, and IBDV especially with older studies, because it is not always clear if these viruses were free of CIAV. For example, Jakowski *et al.* (1970) reported haematopoietic tissue destruction in chickens with Marek's disease (MD), but it was later learned that tumour material harvested from these birds was positive for CIAV (Wellenstein, personal communication, cited by Schat, 2003). In addition to viral infections, coccidial infections have been linked to immunosuppression.

Stress-Induced Immunosuppression

Most environmental factors causing immunosuppression are related to management problems such as inadequate water or food supply, ammonia in the houses, temperature stress, "social interactions within a flock", etc. In addition, the presence of fungal toxins in feed is an environmental stressor. Most of these stressors enhance the production of corticosterone. Selection for high versus low corticosterone concentration in blood plasma can influence the degree of stress-induced immunosuppresion.

It has been recognized for a long time that social stress can exacerbate disease. A classic example is the study by Gross (1972) in which MDV-exposed chickens were kept in a socially stressful environment by moving every day one chicken from one cage into another cage. These chickens developed a higher incidence of tumours than the MDV-infected chickens kept in a low stress environment. The effects of social stress on MD were especially enhanced in birds selected for high plasma corticosterone concentrations. Inoculation with chemicals blocking 11-β hydroxylase, which mediates the conversion of deoxycorticosterone to corticosterone in the adrenal glands, reduced the impact of social stress on MD and other diseases (Gross, 1972, 1989). Inoculation of chickens with corticosterone using pharmacological doses resulted in a rapid lymphoid depletion in thymus, bursa, and spleen (Dohms and Metz, 1991). These authors suggested that bacterial infections (e.g. *Escherichia coli*) may cause stress-type lesions in the bursa similar to corticosterone-induced lymphoid depletion.

The importance of fungal stressors has been reviewed by Bondy and Pestka (2000). Aflatoxin is the best known fungal stressor for poultry but fumonisins and ochratoxins have also been implicated as immunotoxicants in chickens and turkeys. Bondy and Pestka (2000) suggested that the effects are more immunomodulatory than immunosuppressive but the molecular basis for the immunomodulation is poorly understood. Ingestion of aflatoxins can result in decreased antibody responses to T cell-dependent and -independent antigens, decreased macrophage functions, and decreased cell-mediated immune (CMI) responses, but these effects are dose-dependent (Bondy and Pestka, 2000). Chicks hatched from hens fed on diets containing aflatoxins also showed impaired immune functions when tested between 2 and 3 weeks of age

(Qureshi *et al.*, 1998), but it is not known how long after hatching the effects last. These results have important implications and can confound the interpretation of immune dysfunctions in commercial flocks as a confounding factor when virally induced immunosuppression occurs in young birds. Feed contaminated with fumonisins and ochratoxins also impairs antibody responses and macrophage functions, but it is not clear if immunosuppression occurs in chicks hatched from hens fed contaminated diets (Bondy and Pestka, 2000).

Coccidia-Induced Immunosuppression

Two groups of coccidian species have been linked to immunosuppression, *Cryptosporidium baileyi* and *Eimeria* spp., although the evidence is rather limited in both cases. Infection with the latter has been linked to decreased mitogen stimulation responses (Rose and Hesketh, 1984), but antibody responses to T cell-dependent and -independent antigens were not affected (Bhanushali and Long, 1985). *C. baileyi* replicates in the epithelial cells of the bursa of Fabricius and respiratory tract of chickens and is probably more prevalent than diagnostic cases indicate (McDougald, 2003). Oral inoculation of young chickens with high doses of *C. baileyi* caused histopathological lesions in the epithelium and lamina propia of the bursa (Rhee *et al.*, 1997) and decreased antibody responses to T cell-dependent and -independent antigens (Rhee *et al.*, 1998b, 1998c). Titres to infectious bronchitis virus (IBV) and Newcastle disease virus (NDV) were also decreased after inoculation of 2-day-old chicks with *C. baileyi*. (Rhee *et al.*, 1998a, 1998d). On the other hand, Abbassi *et al.* (2000a, 2000b) found that infection with *C. baileyi* neither increased MD nor did it reduce the efficacy of the MDV vaccine strain CVI988. Interestingly, MDV infection before or after challenge with *C. baileyi* aggravated the latter. Likewise, MDV vaccination followed 4 days later with *C. baileyi* challenge caused respiratory lesions within 6 days (see also section MDV).

Virus-Induced Immunosuppression

As mentioned before we will only discuss viruses that may cause immunosuppression independently of clinical symptoms, although the independence may be a temporal feature, as is the case for the tumour-inducing viruses and, depending on the age of challenge, CIAV, and IBDV. The following viruses and their effects on the immune responses will be discussed: IBDV, CIAV, reovirus, and the three tumour-inducing viruses ALV, REV, and MDV. Detailed information on virus replication, pathogenesis, pathology and immune responses for these viruses can be found in several chapters in *Diseases of Poultry*, 11th edition (Saif *et al.*, 2003) and other textbooks on avian diseases.

Infectious Bursal Disease Virus

IBDV, belonging to the Birnaviridae, has been divided into two serotypes, of which only serotype 1 causes immunosuppression and disease in chickens. Several pathotypes are recognized within serotype 1 varying from mild to very virulent. More recently, strains within group 1 have been further subdivided into genetic groups based on restriction enzyme analysis and sequencing. It has been recognized for a long time that serotype 1 strains can cause severe immunosuppression with impaired antibody responses and increased susceptibility to other pathogens, especially when birds are infected before 3 weeks of age (reviewed by Sharma *et al.*, 1994, 2000; Lukert and Saif, 2003).

The genome of IBDV is characterized by a double-stranded RNA (dsRNA) genome consisting of segments A and B. Segment B codes for the viral RNA-dependent RNA polymerase and segment A codes for two structural proteins, VP2 and VP3, an autoprotease, VP4, and a small non-structural peptide, VP5, which partly overlaps with the open reading frame (ORF) coding for VP234 (Lukert and Saif, 2003). The polyprotein VP234, but not the mature VP2, VP3 or VP4, arrests B cell division probably by interfering with the cell cycle, because it does not affect cell viability (Peters *et al.*, 2004). Using a reverse genetics approach, Yao *et al.* (1998) showed

that deletion of VP5 did not prevent virus replication *in vivo* but did prevent the development of bursal lesions suggesting a role for VP5 in the pathogenesis. Interestingly, *in vitro* studies suggest that very early after infection VP5 is anti-apoptotic (Liu and Vakharia, 2006), but during later stages VP5 accumulates in the plasma membrane (Lombardo *et al.*, 2000) and causes apoptosis (Yao and Vakharia, 2001). VP2 has also been linked to apoptosis when expressed alone from transfected RNA (Rodriguez-Lecompte *et al.*, 2005). In summary, the molecular basis for IBDV-induced suppression of the immune response is the result of a complex set of interactions between the different viral proteins and infected B cells.

It has been known since the 1970s that IBDV infection in chicks younger than 3 weeks of age causes severe damage to the bursa of Fabricius with depletion of B cells expressing surface immunoglobulin (Ig)M affecting mostly primary antibody responses. Infection, especially at 1 day of age, also results in a significant decrease of surface Ig-expressing B cells in spleen and peripheral blood lymphocytes but does not affect circulating $CD4^+$ and $CD8^+$ T cells (Hirai *et al.*, 1981; Rodenberg *et al.*, 1994). The damage to the bursa is transient, follicles become repopulated with lymphocytes and tissue architecture is restored, but primary antibody responses remain depressed until at least 7 weeks post-infection (pi), but ultimately also recover. The duration of the recovery process depends on the age of infection and the virulence of the strain (Vervelde and Davison, 1997; Kim *et al.*, 1999). Recently, Withers *et al.* (2005, 2006) described two types of follicles emerging after recovery from IBDV infection in neonatal chicks: small follicles lacking a distinct cortex and medulla and large follicles with rapidly proliferating B cells and a normal structure. B cells in the large follicles were still capable of undergoing gene conversion and may have been derived from small numbers of surviving bursal stem cells. In contrast, the B cells in the small follicles were considered to be derived from more mature B cells that had already undergone gene conversion. These data suggest that the ability to recover from IBDV-induced suppression of antibody production and diversity is based on the proportion of small versus large follicles developing after infection.

In addition to replication in B cells, IBDV can also infect and replicate in macrophages. Khatri *et al.* (2005) found viral RNA and proteins by reverse transcriptase (RT)-PCR and immunohistochemistry, respectively, in bursal macrophages between 1 and 7 days pi. The absolute number of macrophages in the bursa was decreased significantly at 3 and 5 days pi. The actual impact on the immune response of this observation is not clear. Proinflammatory cytokines such as interleukin (IL)-6, IL-1β, and IL-8 are increased at the transcriptional level in the bursa and spleen, while the anti-inflammatory cytokine transforming growth factor (TGF)-β4 is decreased (Kim *et al.*, 1998; Khatri *et al.*, 2005; Eldaghayes *et al.*, 2006). These different groups of authors reported somewhat conflicting results for the production of type I interferon (IFN) and IFN-γ transcription, but these differences could be the result of using different virus strains and/or infecting different age groups. In addition, the production of nitric oxide synthase II – NOS II, previously known as inducible NOS (iNOS) (Bruckdorfer, 2005) – mRNA was also increased in bursal macrophages. Khatri and Sharma (2006) showed that the activation of macrophages is through the p38 mitogen-activated protein kinase (MAPK) and nuclear factor-kappa B (NF$\kappa\beta$) pathway, but it is not clear whether these pathways are activated through specific viral proteins. The alterations in cytokine transcription are compatible with the inflammation in the bursa during the acute infection. However, it is less clear how these changes play a role in IBDV-associated immunosuppression. It will also be important to determine if the changes occur in IBDV-infected macrophages before they undergo lysis or that infection and cytokine deregulation occur in different subpopulations.

Although T cells are not susceptible to infection with IBDV, these cells form an important component of the overall immunopathogenesis of IBDV. There is an influx of $CD4^+$ and $CD8^+$ cells into the bursa between 1 and 10 days pi (Vervelde and Davison, 1997; Sharma *et al.*, 2000). In addition to the proposed role of $CD8^+$ cells in lysing virus-infected cells, bursal $CD8^+$ and $CD4^+$ cells may also contribute to the production of NOS II by IFN-γ production or other soluble factors stimulating NOS II transcription in macrophages and subsequent nitric oxide (NO) production. NO can contribute to the inflammatory lesion development but may also be

involved in downregulation of splenic T cell responses to mitogens, which is associated with the acute phase of IBDV infection (Kim and Sharma, 2000).

In conclusion, IBDV infection causes a complex set of interactions between B cells, macrophages, and T cells leading to destruction and subsequent partial recovery of the bursa and long-lasting suppression of primary antibody responses. Recent studies by several groups have provided a better insight in the immunopathogenesis of IBDV in young birds. However important questions remain. For example, how can we explain the recovery from infection in the face of suppression of primary antibody responses, if neutralizing antibodies are key to recovery? Do we have to postulate that cell-mediated immunity is far more important than previously believed? Another unresolved question is why young birds mostly develop subclinical disease, while infection of older, antibody-negative birds results in clear-cut pathology including haemorrhages. There may be similarities between the situation in birds with IBDV and in mice with influenza virus, where the immune response is responsible for most tissue damage and in which case the damage can be reduced by treatment with TGF-β (Williams *et al.*, 2005).

Chicken Infectious Anaemia Virus

CIAV is a small DNA virus of approximately 25 nm belonging to the Circoviridae, genus *Gyrovirus*. It has a single-stranded covalently closed DNA genome of 2.3 kb, which produces one polycistronic transcript coding for 3 proteins. The virus is extremely resistant to disinfectants and can resist heat treatment at 80°C for 15 min. Due to its ubiquitous presence in chicken flocks, small size, and resistance to physical and chemical treatments, it can be present as a contaminant in other viruses especially if these agents are propagated in embryonated chicken eggs. As such it can become a confounding factor in studies on immunosuppressive properties of other pathogens.

The pathogenesis and immunosuppression caused by CIAV have been reviewed recently (Adair, 2000; Schat, 2003; Miller and Schat, 2004 and additional references within these reviews). Infection of chicks may result in clinical disease by vertical transmission, which occurs when hens first become infected during egg production or horizontal transmission during the first few weeks of age. However, most chicks are protected against early infection by maternal antibodies and clinical disease is not frequently seen. Infection after 3 weeks of age is mostly subclinical but may result in significant immunosuppression. The development of virus-neutralizing (VN) antibodies is essential to curtail virus replication, and immunosuppression caused by IBDV, e.g., has been implicated in prolonged replication of CIAV.

The small genome coding for only 3 proteins requires the infection of dividing cells in order to use the cellular machinery for viral DNA replication. Dividing cells that are susceptible to infection are haemocytoblasts in the bone marrow, T cell precursors in the thymus or dividing T cells in response to antigenic stimulation. VP3 or apoptin is the most important protein for immunosuppression by causing apoptosis of infected haemocytoblasts, resulting in a decrease in erythrocytes, thrombocytes, and granulocytes. The loss of the latter two cell types is important because thrombocytes and granulocytes are both important effector cells during bacterial infections and secondary bacterial infections (e.g. "blue-wing disease") are frequently associated with CIAV-induced immunosuppression. Adair *et al.* (1993) reported that $CD3^+CD8^+TCR\alpha\beta$ spleen cells constitute the major CIAV-infected population in the spleen. The effect of virus replication in these cells is especially important when replication of CIAV occurs at the same time that cytotoxic T cells (CTL) are generated in response to vaccination or infection with a second pathogen. Markowski-Grimsrud and Schat (2003) reported the absence of REV-specific CTL 7 days after birds were co-infected with REV and CIAV, at which time point CIAV was actively replicating, based on real-time quantitative RT-PCR analysis. Because there was no effect of CIAV infection on transcription of IL-2 or IFN-γ at 7 days pi it was suggested that the lack of pathogen-specific CTL was caused by CIAV-induced apoptosis of $CD8^+$ cells during the generation of CTL. In contrast to the effect on CTL, natural killer (NK) cells were not affected by CIAV infection (Markowski-Grimsrud and Schat, 2001). Based on the studies in MSB-1 cells, Peters *et al.* (2006) recently suggested that VP2 may also play a role in immunosuppression

through downregulation of major histocompatibility complex (MHC) class I antigens. The importance of this observation for immunosuppression is difficult to evaluate because the assumption is that CIAV-infected cells will become apoptotic.

The impact of CIAV infection on cytokines has not been well studied and early investigations relied on bio-assays representing the state-of-the-art at the time (reviewed by Miller and Schat, 2004). More recently quantitative RT-assays have been used to investigate the effects of CIAV infection on cytokines in relation to virus replication. Unfortunately, the few published studies have not included the effect of virus replication prior to 7 days pi, when high levels of virus replication occur (Markowski-Grimsrud and Schat, 2003; van Santen *et al.*, 2004). At that time, immunosuppressive effects are already evident with impairment of macrophages (McConnell *et al.*, 1993) and CTL (Markowski-Grimsrud and Schat, 2003), but IFN-γ, IL-2 and IL-1β mRNA levels were not affected (Markowski-Grimsrud and Schat, 2003). Clearly, additional studies using quantitative RT-PCR assays or enzyme-linked immunoassay (ELISA) are needed to determine the impact of CIAV infection on cytokines starting at 2–3 days pi, because viral antigens can be detected in lymphoid tissues and bone marrow as early as 3–4 days pi (Smyth *et al.*, 1993). Interestingly, the damage to the thymus and bone marrow was quite extensive between 3 and 12 days pi though relatively few cells in these organs were positive for viral antigens. The collapse of the thymus is probably the result of damage to the cytokine network needed for T cell maturation. Detailed studies on early cytokine changes during CIAV infection are essential to gain an understanding of the subsequent immunosuppression.

Reovirus

Avian reoviruses belong to the *orthoreovirus* genus of the Reoviridae, which have a genome consisting of 10 dsRNA fragments. Avian reovirus infections can cause tenosynovitis in chickens and other diseases, or result in a subclinical infection. Although horizontal transmission is the main route for infection, egg transmission may occur infrequently (Menendez *et al.*, 1975). Infections with pathogenic, but not non-pathogenic, strains have been associated with depletion of lymphoid cells in the bursa and thymus and decreased serological responses to inactivated NDV (Sharma and Fredericksen, 1987). However, Montgomery *et al.* (1986) were unable to find a significant impact of reovirus infection on antibody responses to NDV, sheep red blood cells (SRBC), and *Brucella abortus* antigen, although a decrease in relative bursa weight and some lymphocyte depletion in the bursa were noted. Reovirus infection decreased the responses of peripheral blood monocytes and splenocytes to mitogens at 7 days pi but not afterwards (Montgomery *et al.*, 1986; Sharma *et al.*, 1994; Pertile *et al.*, 1995, 1996). The latter group showed that removal of plastic-adherent cells primed to produce NO restored mitogen responsiveness of T lymphocytes to some degree and suggested that suppressor macrophages were responsible for the immuno-suppression. Recent work with mammalian species suggests that the regulation of macrophage activation is rather complex. For example, IL-13 may be responsible for the induction of macrophages producing NO (Sinha and Garg, 2000) while IL-10, through the suppressor of cytokine signaling-3 (SOCS3), may downregulate the inflammatory responses of macrophages (Qasimi *et al.*, 2006). The S1 genome segment of avian and mammalian reovirus, which has been linked to immunosuppression (Garzelli *et al.*, 1985), codes for 3 open reading frames (ORF) two of which are non-structural proteins. These two proteins have pleiotropic functions, many of which interfere with cellular signaling pathways (Liu *et al.*, 2005). Additional research on the interactions of these proteins with cytokine pathways will be important to more fully understand reovirus-induced immunosuppression in chickens.

Tumour Viruses

The three tumour viruses associated with lymphoid tumours, MDV, ALV, and REV, are discussed in more detail in Chapter 19. In this chapter, only the immunosuppressive aspects are reviewed. Extensive reviews on the diseases caused by these viruses are available (Fadly and Payne, 2003; Witter and Fadly, 2003; Witter and Schat, 2003).

Marek's Disease Virus

MDV, an alphaherpesvirus causing tumours of T lymphocytes in chickens, has attracted the attention of virologists and immunologists, since highly successful vaccines protecting against the disease became available during the early 1970s. Since then, the virus has evolved from virulent to very virulent (vv) and more recently to vv+ pathotypes (Witter, 1997). The increase in pathogenicity not only increased the incidence of tumours but was also linked to some new neurological syndromes (Gimeno *et al.*, 2002). In addition, the vv+ strains caused more severe damage to the lymphoid organs than virulent and vv pathotypes (Calnek *et al.*, 1998) probably leading to increased immunosuppression.

Immunosuppression caused by MDV has recently been reviewed by Schat and Markowski-Grimsrud (2001) and Schat (2004) and in this chapter we will only present the most important aspects of MDV immunosuppression and immunoevasion (see section immunoevasion). MDV-associated immunosuppression is often divided into an early immunosuppression phase during the cytolytic infection and a late immunosuppression phase, when virus is reactivated and tumours develop. One of the key characteristics of the early immunosuppression is the destruction of lymphocytes in the lymphoid organs during the first 2 weeks of infection causing severe atrophy of the thymus and bursa of Fabricius; this may be permanent or transient depending on the pathotype of MDV. The model for the pathogenesis of MD, as originally outlined by Calnek (1986) and Schat (1987) and recently reviewed by Calnek (2001), provides the basis for the understanding how MDV causes both B and T cell depletion. Briefly, chickens become infected through cell-free virus present in feather-follicle dander. Virus is transferred to the lymphoid organs probably through macrophages and replicates first in B lymphocytes causing a productive–restrictive infection in which cell-associated but not cell-free MDV is produced and the infected cells are destined to die. This phase is often referred to as the cytolytic phase. As a consequence of the production of viral antigens and subsequent immune responses, T cells become activated expressing MHC class II antigens and other activation markers. In contrast to resting T cells, activated T cells are susceptible to MDV infection and become infected. Normally, MDV establishes a latent infection in activated T cells through poorly understood mechanisms (Morgan *et al.*, 2001b), although cytokines most likely play a role in the process (Schat and Markowski-Grimsrud, 2001). Depending on the pathotype of MDV and the genetic resistance of the host, latency may be permanent, temporarily followed by a secondary cytolytic cycle, additional immunosuppression and tumour development, or absent with continuous virus replication and, frequently, early mortality.

As early as 3 days pi, the expression of IFN-γ is upregulated (Xing and Schat, 2000), which led Schat and Markowski-Grimsrud (2001) to suggest that this may upregulate the expression of IL-8 receptors. The upregulation of IL-8 receptors is likely an essential step in the transfer of cell-associated MDV from B cells to activated T cells, allowing viral IL-8 (VIL-8) produced during the lytic infection (Parcells *et al.*, 2001) to attract activated T cells. The increased cytolytic infection, and thus increased damage to the lymphoid organs associated with some of the vv+ pathotypes, may be caused by a more effective transfer of MDV from B to T cells due to increased levels of vIL-8 production combined with the inability to establish latency after 7 days pi. Jarosinski *et al.* (2003) reported that vv+ strains produced higher levels of vIL-8 than less virulent strains, but this is likely the consequence of increased virus replication (Yunis *et al.*, 2004) rather than an intrinsic ability to enhance vIL-8 production. The importance of vIL-8 for the cytolytic infection is further shown by the fact that tissue culture-attenuated strains have no or very low vIL-8 transcript levels (Jarosinski *et al.*, 2003) and that deletion of vIL-8 (Cortes and Cardona, 2004; Cui *et al.*, 2005) or deletion of exon 1 of vIL-8 (Jarosinski and Schat, 2007) result in attenuation of the cytolytic infection. The reasons for the continued replication of vv+ MDV instead of establishment of latency have not been elucidated. It is of interest to note that vv+ MDV causes a highly significant upregulation of proinflammatory cytokines as early as 4 days pi in spleen and brain tissues (Jarosinski *et al.*, 2005; Careem *et al.*, 2006). It is certainly feasible that the distortion in cytokine profile impacts on the production of cytokines involved in the induction and/or

maintenance of latency (Volpini *et al.*, 1995) with the consequence of a prolonged cytolytic infection leading to more profound immunosuppression.

Apoptosis is the most likely mechanism responsible for cell death during the cytolytic infection in the lymphoid organs (Morimura *et al.*, 1996, 1997) affecting $CD4^+CD8^+$ thymocytes. What needs to be resolved is: do MDV-infected thymocytes become apoptotic or does MDV-induced cytokine deregulation affecting thymocyte maturation cause apoptosis of non-infected cells and a collapse of thymus architecture (Schat, 2004)? The latter is certainly possible, based on the recent evidence that cytokines can be deregulated especially after infection with vv+ strains. MDV infection can also cause apoptosis in $CD4^+$ peripheral T cells, but $CD8^+$ T cells are apparently not affected (Morimura *et al.*, 1995). The reasons for the latter are not clear because $CD8^+$ T cells can be infected with and transformed by MDV (Baigent *et al.*, 1998; Calnek *et al.*, 1989). Thus far, it has not been resolved which MDV gene products are responsible for the immunsuppression. It is likely that the SORF2 gene plays a role, based on two studies using opposing experimental strategies. Deletion mutants lacking several ORF, including SORF2, caused decreased cytolytic infection without preventing tumour formation (Parcells *et al.*, 1995). Upregulation of SORF2 expression through the insertion of the REV long terminal repeat (LTR) resulted in enhanced cytolytic infection in the absence of tumour development (Witter *et al.*, 1997).

In addition to the destruction of lymphoid tissues, MDV also induces immune suppression through activation of macrophages. These macrophages were able to inhibit mitogen stimulation of T cells obtained from non-infected chickens (Lee *et al.*, 1978). Schat and Markowski-Grimsrud (2001) suggested that this inhibition was likely the consequence of MDV-induced NO production by the macrophages and was protective rather than immunosuppressive by reducing the pool of activated T cells. The concept of immunosuppression around 7 day pi is conflicting because on the one hand there is destruction of lymphoid tissue, potential immunoevasion and the activation of "suppressor" macrophages, while at the same time CTL against several MDV proteins are present (Schat and Markowski-Grimsrud, 2001; Markowski-Grimsrud and Schat, 2002). In contrast with the more classical strains, vv+ strains may actually infect macrophages (Barrow *et al.*, 2003a) and macrophages have been associated with MDV lesions in the brain (Barrow *et al.*, 2003b; B.L Njaa, K.W. Jarosinski and K.A. Schat, unpublished data). The importance of these observations for immunosuppression is not clear; more likely it will be part of the enhanced proinflammatory response in the brain (Jarosinski *et al.*, 2005).

Late immunosuppression can be caused by lytic infection of lymphoid cells upon reactivation of MDV from latency, causing similar effects on the immune system as during the early lytic infection. In addition, tumour cells may cause immunosuppression (reviewed by Schat, 2004). However, tumour-induced immunosuppression is directly the consequence of a clinical disease and will not be discussed here.

MDV vaccine strains can also cause immunosuppression. Friedman *et al.* (1992) showed that vaccination with SB-1 (a serotype 2 MDV strain) together with herpesvirus of turkeys (HVT) or CVI988 (a serotype 1 vaccine strain) caused a significant decrease in B lymphocyte activation and antibody production after *in vitro* stimulation with *Salmonella typhimurium* or bovine serum albumin (BSA) inoculation, respectively. *E. coli*-caused mortality was increased in vaccinated chicks independently of the MDV vaccine used, when challenged at 11 or 14 days post-vaccination, but only occurred in chicks vaccinated with CVI988 when these were challenged at 21 days post-vaccination. Islam *et al.* (2002) also reported that HVT did not cause increased mortality when chickens were challenged with *E. coli* at 28 days post-vaccination, although between 3 and 10 days HVT caused a significant decrease in B and T lymphocyte numbers. Vaccination with CVI988 also precipitated lesions when chicks were challenged with *C. baileyi* 4 days post-vaccination (see section Coccidia-Induced Immunosuppression). The importance of vaccine-induced immunosuppression is not clear in commercial flocks and is far less important than the protection against MD challenge. The possibility that *in ovo* vaccination with HVT might induce tolerance was investigated by Zhang and Sharma (2003). Inoculation between 0 and 14 days of embryonation-induced tolerance to HVT but not to BSA. In contrast

vaccination at 18 days of embryonation, which is the approximate time for *in ovo* vaccination, did not induce tolerance to HVT.

ALV and REV

ALV and REV belong to the Retroviridae. ALV are classified as exogenous or endogenous (or subgroup E) viruses and the former are further divided in subgroups A, B, C, D, and J. Subgroups A–D are associated with infection of Leghorn type chickens, but subgroups C and D are of little practical importance for the poultry industry. ALV-J was first described during the late 1980s and is mostly associated with meat-type birds. ALV and REV can also be divided into defective viruses and non-defective (nd) or helper viruses. The defective viruses have often acquired cellular *onc* genes and cause a rapid onset of tumours and death. These viruses have little importance for immunosuppression due to the rapid mortality. The nd viruses can also cause tumours, but the transformation occurs through activation of cellular *onc* genes by the viral LTR.

REV and exogenous ALV can be transmitted congenitally or horizontally. Chicks hatched from congenitally-infected eggs are tolerant and unable to produce VN antibodies to the virus. These chickens will be viraemic, antibody negative (V+A−) and are likely to develop tumours or in the case of REV tumours and/or runting and stunting. Horizontal transmission during, or shortly after, hatching can also result in tolerance. This risk is enhanced in chicks when subgroup E or glycoprotein (gp)85 of subgroup E is expressed during embryonal development (Smith *et al.*, 1991) or after IBDV infection at 1 day or 6 weeks of age. IBDV infection decreased the frequency of VN antibody-positive chickens, and increased virus shedding and viraemia levels significantly, although these effects were dependent on the genetic background of the birds. In contrast infection with REV at 1 day of age or MDV at 2 weeks of age had no significant impact on ALV shedding, viraemia, and antibody development (Fadly *et al.*, 1985). The induction of tolerance after congenital infection is virus specific and is not a general inability to produce antibodies against other pathogens than ALV or REV. For example, chickens congenitally infected with a specific strain of ALV-A were able to produce antibodies to other subgroup A strains (Meyers, 1976) and influenza virus, but were partly tolerant to ALV-B (Meyers and Dougherty, 1971).

The importance of ALV as an immunosuppressive virus is not clear and probably depends on the subgroup. Fadly *et al.* (1982) reported that congenital infection with RAV-1, a subgroup A virus, did not suppress antibody responses, mitogen stimulation, or the phytohaemagglutinin (PHA) skin test, even in birds developing tumours. Rup *et al.* (1982) also reported that subgroup A viruses did not affect mitogen responsiveness of spleen lymphocytes. Likewise, several groups have shown that ALV-J infection does not affect cell-mediated and humoral immune responses to a significant degree. However, most assays for immune responses and resistance to other diseases were marginally lower in ALV-J infected than in control chickens (Stedman *et al.*, 2001; Landman *et al.*, 2002; Spackman *et al.*, 2003), suggesting, but not proving, that immunosuppression may be associated with ALV-J infection.

In contrast to ALV-A and -J, subgroup B viruses have been linked to suppression of CMI responses. *In ovo* infection with MAV-2(O) resulted in severe damage to lymphoid organs, suppressed antibody responses and mitogenic responsiveness (Hirota *et al.*, 1980). Rup *et al.* (1982) and Cummins and Smith (1987) showed that MAV-2(O) caused a temporary dysfunction of macrophages suppressing the responses of spleen cells to mitogens. The depressed responses were present as early as 3 days pi and lasted until 21 days pi. Macrophage dysfunction reduced the ability to clear bacteria such as *Listeria monocytogenes* (Cummins *et al.*, 1988). A second subgroup B virus, avian erythroblastosis virus, an acute transforming, defective virus, and its associated helper virus, also suppressed T cell responses to mitogens, but in this case the suppression was directly related to T cells and not macrophages (Rao *et al.*, 1990).

REV infections have frequently been linked to depressed humoral and CMI responses. At least theoretically, this could have important practical consequences, because REV infection has often been linked to the use of contaminated MD vaccines (Koyama *et al.*, 1976;

Jackson *et al.*, 1977). Fowlpox viruses (FPV) can carry replication competent REV genomes, which may be expressed *in vivo* (Diallo *et al.*, 1998) potentially causing immunosuppression. REV infection decreased antibody responses to HVT, MDV, and NDV (e.g. von Bülow, 1977; Yoshida *et al.*, 1981). In contrast, Fadly *et al.* (1985) and Witter *et al.* (1979, 1981) found only decreased primary antibody responses to SRBC and *Brucella abortus*, and no effect on MDV antibodies, although protective immunity to MD was decreased and ALV infection levels were increased in a commercial line. Embryo inoculation with the T strain, but not with the Cornell C strain (CS) strain, impaired also the secondary immune responses to SRBC and *Brucella abortus*. The effect of REV infection on MDV was questioned by Buscaglia *et al.* (1989) because REV infection did not impair the establishment of MDV latency or reactivation from latency. These authors suggested that REV may actually reduce the pool of susceptible cells for MDV replication, which would be compatible with the REV-associated atrophy of the lymphoid organs (Witter *et al.*, 1981). The lymphoid atrophy is strain specific and is caused by a combination of *env* and *gag* genes (Filardo *et al.*, 1994).

The effects of REV infection on cell-mediated immunity have been studied by several groups mostly using mitogen stimulation assays (e.g. Witter *et al.*, 1979; Walker *et al.*, 1983; Buscaglia *et al.*, 1989). The immunosuppressive effects were linked to the development of suppressor cells by Rup *et al.* (1982, 1979). Unfortunately, there are no recent reports characterizing these cells or their mode of action. The importance of this type of cell-mediated suppression needs to be further analyzed especially in view of the development of REV-specific $CD8^+$ CTL starting at 6 days pi with n.d. REV (Lillehoj *et al.*, 1988; Weinstock *et al.*, 1989).

Mechanisms of Immunosuppression

In the previous section, three broad categories causing immunosuppression were identified: corticosteroids in relation to stress, apoptosis and/or necrosis of lymphoid cells and virus-induced changes in the regulation of immune responses. In many instances, the actual molecular interactions between virus proteins and host cells are poorly identified. In order to use cytokines as adjuvants to improve vaccines as suggested by Asif *et al.* (2004), future research needs to be focused on a better understanding of the interactions between viruses and cytokine regulation.

Corticosteroids and Stress-Induced Immunosuppression

Dohms and Metz (1991) indicated that the mechanisms of stress-induced immunosuppression were poorly understood especially in birds. The common pathway for stressors involves the hypothalamic-pituitary-adrenal (HPA) axis and results in the release of glucocorticoids, which are immunosuppressive for many species including chickens. Treatment of chickens with corticosterone or related glucocorticoids results in lymphopenia and atrophy of the lymphoid organs. Pruett (2001) suggested that the interactions between glucocorticoids and the immune system are more complex and, depending on the duration of the stress, can enhance or suppress or have no effect on immunological variables in mammalian species. Measuring several immunological parameters, El-Lethey *et al.* (2003) compared the effects of treating chickens with corticosteroids with the effects of lack of foraging materials. Interestingly, antibody titres against SRBC and tetanus toxoid were decreased after both treatments, but antibodies to human serum albumin were not influenced by either treatment. In addition, CMI responses such as cutaneous response to PHA and delayed-type hypersensitivity (DTH) responses to mycobacterium antigen were decreased with both treatments. It is also noteworthy that the lack of foraging materials did not enhance plasma corticosterone levels significantly. In conclusion, stress may result in immunosuppression but the actual mechanisms remain elusive.

Apoptosis and/or Necrosis

Virus replication in lymphoid cells is the major cause of cell death for IBDV, CIAV, reovirus, MDV and to some degree REV. In most of these infections, apoptosis is the cause of cell death

although necrotic lesions have been reported for MDV (Witter and Schat, 2003). The induction of apoptosis by VP5 and VP2 of IBDV was mentioned before, but it is not clear how these proteins actually interact with the apoptotic pathway. Liu and Vakharia (2006) showed that IBDV infection *in vitro* activates effector caspase 3 and the initiation caspase 9 as well as NFκB resulting in apoptosis late in the infective cycle. These authors suggested that NFκB may be activated through the accumulation of reactive oxygen species as has been shown for reovirus-induced apoptosis.

CIAV provides another example for viral induction of apoptosis (Noteborn, 2004). VP3 (or apoptin) is the major viral protein inducing apoptosis although VP2 may also be a minor cause of apoptosis. Early after transfection of MSB-1 cells with a VP3-expressing plasmid, VP3 is present in the cytoplasm and nucleus as fine granular structures. At the time that cells become apoptotic VP3 forms discrete nuclear structures. The carboxy terminus is positively charged and, based on work with deletion mutants, it is suggested that the positive charge is important for the interaction with the nuclear DNA. Noteborn (2004) suggested that this may lead to alterations in the supercoiled organization of the cellular DNA causing apoptosis or alternatively that VP3 acts a transcriptional regulator of genes involved in the apoptotic process.

Although MDV induces apoptosis, little is known about the viral proteins and mechanisms involved in the induction of apoptosis. Thus far, no proteins preventing or inducing apoptosis have been found in MDV, although Kingham *et al.* (2001) identified in the related HVT an ORF coding for a protein with significant similarity to the putative quail anti-apoptotic gene NR-13. Ross (1999) speculated that the phosphorylated polypeptide pp38 was responsible for apoptosis in MDV. However, Li *et al.* (2006) showed that induction of pp38 expression under control of an inducible promoter did not induce apoptosis in the quail cell line QT35.

Virus-Induced Changes in the Regulation of Immune Responses

Thus far, there are few studies describing quantitative changes in cytokine production as a consequence of virus infections in chickens. It is not clear how many of the changes are actually related to immunosuppression rather than changes associated with the induction of immune responses. It is clear from the work by Jarosinski *et al.* (2005) using vv+ MDV strains that strong proinflammatory responses may cause pathology. This observation has important consequences for genetic selection for MD resistance, because the resistant N2a line had a very strong proinflammatory response causing neurological disease with significantly higher levels of cytokine and NO production in the brain than in susceptible P2a chickens. Additional studies on cytokine deregulation leading to immunosuppression are urgently needed using real-time RT-PCR assays and preferably ELISA to examine the impact on transcription and actual production of cytokines.

One of the common immunosuppressive effects described for MDV, reovirus, IBDV, and REV infections is the generation of "suppressor" macrophages. These cells suppress T lymphocyte blastogenesis using mitogen responses and are frequently detected between approximately 3 and 15 days pi. Removal of these cells restores the responsiveness of the T cells (e.g. Lee *et al.*, 1978; Rup *et al.*, 1979; Pertile *et al.*, 1995). Interestingly, this immunosuppression has been linked to NO production by macrophages. NO is a highly versatile molecule with immunosuppressive as well as anti-tumour, anti-bacterial, and antiviral activity. NO can influence gene expression probably through the NFκB pathway and, at high levels, can be part of proinflammatory pathology (reviewed by Bruckdorfer, 2005).

IMMUNOEVASION

Introduction

As we have learnt more about the molecular interactions between viruses and their hosts, it has become apparent that most, if not all, viruses have to employ immunoevasion mechanisms to survive even the innate immune responses of the host. Those viruses with a persistent lifestyle

also have to deploy a spectrum of subtle, refined mechanisms to survive in the face of potential acquired immune responses. The factors involved in immunoevasion were most easily identified in the large DNA viruses (poxviruses and herpesviruses). With the exception of the molluscipoxviruses, which have no direct relatives in birds but which have some similarities with the avian poxviruses, poxviruses cause acute infections and so their immunoevasion mechanisms only need to counter (or are only capable of countering) the innate immune responses, and possibly delay the acquired responses. The herpesviruses are able to become latent, a state that requires the expression of very few genes. They do, however, appear to be able to prevent the induction of acquired responses, even though the immune system is regularly open to stimulation by episodic, localized reactivation of latent virus. The recognition that large complicated viruses needed to evolve specific mechanisms to counter innate immune responses arguably drove the suspicion that the smaller, "simple" viruses (often with an RNA genome) must have evolved equivalent mechanisms to survive the same pressures. There are now several examples, particularly in the paramyxoviruses, of small accessory proteins of RNA viruses, or of multifaceted proteins, playing a role in immunoevasion, particularly in suppressing the type-I IFN pathway. The relative dearth of reagents available for many aspects of avian biology means that detailed study of the mode of action of many of the immunoevasion mechanisms of avian viruses remains woefully behind that of mammalian viruses. Indeed many of the mechanisms remain presumptive, but the situation should improve with the recent derivation of the draft chicken genome sequence. Recognition of the importance of avian viruses as emerging zoonotic agents, for instance West Nile virus and H5N1 avian influenza virus (AIV), is also likely to drive much more work in this area. We will consider immunoevasion mechanisms, both known and postulated, of avian herpesviruses, poxviruses, orthomyxoviruses, paramyxoviruses, and reoviruses, as well as reviewing prospects for future work on avian adenoviruses and coronaviruses, orthomyxoviruses, and paramyxoviruses.

Immunoevasion Mechanism of the Avian Herpesviruses

Study of the biology of MDV teaches us that it uses at least two strategies of immunoevasion, though no specific mechanisms have as yet been attributed to these observations. Morimura *et al.* (1997) showed that MDV infection downregulates transcription of CD8. Hunt *et al.* (2001) reported that MHC class I expression was downregulated in two cell lines, MDV-infected OU2 cells and in MDV-transformed MSB-1 cells treated with 5-bromo-2′deoxyuridine, most likely through a block in the transport of the MHC molecules to the cell surface. Morgan *et al.* (2001a), using microarray analysis, found that MDV upregulated MHC class I, but this is likely to be IFN driven. A mechanism that may favour survival of MDV in its latent state is promotion of the survival of MDV-transformed tumour cells. Whether this is achieved directly by enhanced expression of chicken CD30, previously identified as the AV37 antigen (Burgess *et al.*, 2004), or by the downregulation of CD28 seen in cells over-expressing CD30 (Burgess and Davison, 2002) is not clear, nor is the actual viral mechanism of achieving these perturbations in cell surface marker expression.

The herpesviruses have proved a rich hunting ground for those seeking the mediators of immunoevasion, hereafter referred to as immunomodulators, and the avian herpesviruses, such as MDV, prove no exception. The most interesting immunomodulator found in MDV is the IL-8 mimic (vIL-8), identified initially by its sequence homology with host IL-8. More informatively referred by its synonym, eotaxin, IL-8 is a chemokine capable of attracting eosinophils. Deletion of vIL-8 (Cortes and Cardona, 2004; Cui *et al.*, 2005) or exon 1 of vIL-8 (Jarosinski and Schat, 2007) from the RB-1B strain of MDV resulted in a lower frequency of birds with tumours and smaller, less invasive tumours. This suggests that vIL-8 affects virus replication rather than tumorigenesis. The possibility of immunomodulatory functions for vIL-8 cannot be eliminated but the discovery that it can also be expressed as a fusion protein with the MDV oncoprotein Meq presents the likelihood of a more complicated situation (Anobile *et al.*, 2006).

Immunoevasion Mechanism of the Avian Poxviruses

The avian poxviruses comprise one genus (the *Avipoxvirus* genus) of the Chordopoxvirinae subfamily. The other seven genera comprise only mammalian poxviruses. Recent phylogenetic studies, based on only a couple of conserved genes, reveal that the avipoxviruses show considerable diversity, equivalent to that observed between the *Capripoxvirus*, *Suipoxvirus*, *Yatapoxvirus,* and *Leporipoxvirus* genera of the mammalian poxviruses. They appear to form three major clusters (or clades) of related viruses, one broadly related to FPV, one to canarypox virus (CNPV) and the other comprising the psittacine poxviruses (Jarmin *et al*., 2006). Various phylogenetic studies have indicated that the mammalian poxvirus most closely related to the avipoxviruses is the human molluscum contagiosum virus, with which avipoxviruses share many common features of molecular biology, including key aspects of gene complement and organization, as well as aspects of pathogenesis.

The avipoxviruses have some of the largest DNA viral genomes, up to more than 300 kbp and encoding up to 300 genes. The complete genome sequences of pathogenic and attenuated vaccine strains of FPV (Afonso *et al*., 2000; Laidlaw and Skinner, 2004) and of a pathogenic strain of CNPV have been determined (Tulman *et al.*, 2004). Avipoxviruses cause diseases ranging in severity and mortality from relatively mild cutaneous infections, e.g. fowlpox in chickens, through the more severe diphtheritic infections (also seen in some cases of fowlpox) to disseminated systemic or pneumonia-like infections with high mortality, such as in CNPV infections. The nature of the infection is probably related to host-virus adaptation, in which immunoevasion mechanisms and especially immunomodulators play an important role. The less severe infections probably reflect long-standing adaptations of virus and host, whereas the more severe infections probably reflect more recent introductions of viruses to novel hosts.

The derivation of the first sequences brought with it the surprise that FPV encoded no obvious candidates for modulators of the type I IFN or IFN-γ pathways, nor of the antiviral effectors which these pathways induce (Tulman *et al*., 2000, 2004; Laidlaw and Skinner, 2004). This was in contrast to the mammalian poxviruses, which encode: dsRNA binding proteins (e.g. E3 of vaccinia virus); mimics of eIF2α, the substrate for activated dsRNA-dependent protein kinase, PKR (e.g. vaccinia virus K3) and secreted, soluble binding proteins for IFN-I and IFN-γ. However, FPV and CNPV appear to encode mimics of TGF-β; and CNPV encodes an IL-10-like protein. Assuming they act as agonists, both of these candidate immunomodulators are predicted to downgrade the host's inflammatory responses by stimulation of T regulator cells, thereby protecting the virus-infected cell.

Two groups independently predicted that two different FPV genes (fpv073 and fpv214) would encode candidate IL-18 binding proteins (Tulman *et al.*, 2000; Laidlaw and Skinner, 2004). Only one of the predicted genes (fpv214) encoded a protein with a conserved IL-18 binding motif and, interestingly, a knockout of this gene in a recombinant FPV expressing IBDV VP2 resulted in an enhanced CMI response against IBDV, when chickens vaccinated with the recombinant were challenged with IBDV. The enhancement was comparable to, but less dramatic than, the enhancement observed when the VP2 recombinant FPV co-expressed chicken IL18, whether fpv214 was intact or not (Eldaghayes *et al.*, 2008).

The avipoxviruses also encode multiple chemokine-like molecules (e.g. fpv060, fpv061, fpv116, and fpv121). These appear to be secreted, although as yet no biochemical activity has yet been attributed to any of them, partly due to the lack of identified chicken chemokine and chemokine receptor reagents (Jeshtadi *et al*., 2005). It is therefore unclear whether they will behave as agonists or antagonists and whether they will target *bona fide* chemokine receptors or similar receptors for other host ligands. Avipoxviruses also encode serpentine molecules with seven transmembrane segments resembling G-protein-coupled receptors. These proteins, such as fpv021, fpv027, and fpv206 are candidate chemokine receptors (Tulman *et al.*, 2000). Whether they actually bind host chemokines or other host ligands, and whether they are signaling-competent, or merely decoy receptors remains to be tested when chicken chemokine reagents become available.

Although no gene encoding IFN-γ-binding protein was predicted from the sequence, a binding protein was identified biochemically by its interaction with recombinant tagged IFN-γ. It proved to be the product of fpv016 (Puehler *et al.*, 2003). Type I IFN-binding proteins are not predicted and it remains to be seen if alternative approaches will identify genes encoding such proteins. The lack of obvious mechanisms for evasion of the type I IFN responses is intriguing, particularly as we have evidence that FPV is resistant to recombinant chicken type I IFN at a concentration over 1000-fold higher than is required to inhibit vaccinia virus strain MVA (Pollitt, 1997; M. Skinner, unpublished data).

The avipoxvirus genomes encode many as yet unassigned proteins, which might function in immunoevasion. For instance, one feature that distinguishes avipoxviruses from mammalian poxviruses is that the former encode several families of related proteins. Most notable is the family of proteins containing multiple ankyrin repeats. These repeats are found in many types of host proteins and their presence normally denotes a protein–protein interaction. Up to 30 proteins of this family are encoded by the avipoxviruses – we have no idea of their targets but presume they are likely to be host proteins and that some at least will probably play a role in immunoevasion.

Immunoevasion Mechanism of the Avian Orthomyxoviruses

Viruses of the Orthomyxoviridae family are enveloped viruses enclosing eight segments of negative strand RNA. The best known are members of the *Influenzavirus A* genus, mostly as a consequence of their role in human pandemics. As is now widely appreciated since the emergence of the H5N1 strain, influenza A viruses are primarily an infection of birds, originating in waterfowl, and readily passing to a wide range of wild and domestic birds.

It was using influenza virus infection that IFN was discovered in 1957 (Lindenmann *et al.*, 1957). Subsequently, it was one of the first viruses for which a resistance mechanism to IFN (activation of host protein p58) was elucidated (Lee *et al.*, 1990). Since 1997 and the emergence of the highly pathogenic H5N1 AIV capable of causing high mortality in humans, NS1 has been identified as the major viral IFN-resistance protein (Garcia-Sastre *et al.*, 1998; Hatada *et al.*, 1999; Bergmann *et al.*, 2000; Talon *et al.*, 2000). Moreover, a determinant of virulence of H5N1 (D92E) has been located on NS1 (Seo *et al.*, 2004). These studies have been performed in the context of mammalian IFN responses, this being appropriate because of the clinical threat and also practical because the avian IFN response is not fully understood and there is a lack of avian reagents.

Perhaps because of the lack of biochemical assays and reagents, one of the few direct studies of the interaction between AIV and avian IFN was performed with live virus (Marcus *et al.*, 2005). The study concluded that there was considerable heterogeneity within and between virus populations in their ability to induce and resist avian IFN I. The heterogeneity was ascribed to the presence in the population of subpopulations that had packaged multiple genome segments. It was presumed that those particles which had packaged multiple segments encoding IFN resistance protein(s) displayed higher resistance, although this characteristic was not necessarily inherited. Such heterogeneity illustrates one of the potential complications of using live-recombinant influenza viruses to dissect out the role of particular molecular determinants of IFN resistance.

Studies with the mammalian system have revealed that NS1 is a very complex, multifunctional protein. It has at least three different major roles: (i) binding dsRNA to block the IFN response, (ii) inhibiting host gene expression by preventing mRNA splicing and nuclear export and (iii) enhancing viral mRNA translation. All of these roles merit further investigation in mammalian and avian hosts.

Resistance of influenza virus (influenza A virus unless otherwise specified) to IFN was initially believed to be primarily due to activation of a cellular inhibitor, p58(IPK), of PKR (Lee *et al.*, 1990). The mechanism of activation is still not known. Another mode of action involved the ubiquitin-like host protein, ISG15, which is one of the most predominant proteins induced by type I IFN (Bazzigher *et al.*, 1992). Influenza B virus induces ISG15 strongly but a specific

region of the influenza B virus NS1 protein (NS1B), which includes part of its effector domain, blocks the ability of ISG15 to become covalently linked to its target proteins by inhibiting its UBE1L-mediated activation. The influenza A virus NS1 protein does not bind ISG15, but inhibits its synthesis (Yuan and Krug, 2001). Subsequently, however, NS1 was shown to play a major role in resistance to IFN. Specifically, NS1 mutants were able to replicate only in IFN-defective cell lines (Garcia-Sastre *et al.*, 1998). NS1, normally 230 amino acids long in influenza A virus, is encoded by virus RNA segment 8. The amino terminus (73 residues) of NS1, which dimerizes (Wang and Krug, 1996), is capable of binding dsRNA (Hatada and Fukuda, 1992), albeit at relatively low affinity (Yuan and Krug, 2001), thereby preventing activation of the dsRNA-dependent protein kinase, PKR (Lu *et al.*, 1995). NS1 also binds polyadenylated RNA, inhibiting nuclear export of mRNAs (Qiu and Krug, 1994), and a stem bulge in U6 snRNA, inhibiting pre-mRNA splicing *in vitro* and *in vivo* (Qiu *et al.*, 1995). Both of these activities can downregulate expression of cellular genes. It can also prevent IFN production both by binding to IRF-3, blocking its kinase-mediated activation (Talon *et al.*, 2000), and by blocking NFκB activation (Wang *et al.*, 2000). Although expression of NS1 alone was reported to induce apoptosis in MDCK and HeLa cells (Schultz-Cherry *et al.*, 2001), in the context of a viral infection of mammalian or avian cells, its IFN-regulatory activity makes it anti-apoptotic (Zhirnov *et al.*, 2002). NS1 selectively enhances translation of viral but not cellular mRNAs by binding eIF4GI, PABP1, and the 5′ UTRs of vmRNAs (Aragon *et al.*, 2000; Burgui *et al.*, 2003). It is interesting that the region of NS1 (aa 81-131) binding eIF4GI spans the location of the known virulence mutation (aa 92) and that the region of PABP1 to which NS1 binds is not conserved evolutionarily. The C terminus, or effector domain, downregulates formation and export of cellular mRNAs, by binding to the 30 kDa subunit of CPSF and to PABII (Nemeroff *et al.*, 1998; Chen and Krug, 1999, 2000). The dsRNA-binding activity of NS1 can be abrogated by mutating 2 basic residues (R38 and K41 to A). In MDCK cells, virus thus mutated (Donelan *et al.*, 2003) failed to inhibit IFN-β and replicated to lower titres. On passage, a better replicating virus emerged with an S42G mutation that did not improve dsRNA binding, but which had intermediate virulence in mice.

Immunoevasion Mechanism of the Avian Paramyxoviruses

The Paramyxoviridae family of negative sense RNA viruses, which includes measles virus, has two well-known avian members: NDV (member of the *Avulavirus* genus) and turkey rhinotracheitis virus or avian metapneumovirus (member of the *Metapneumovirus* genus).

There has recently been considerable study of the mechanism by which paramyxoviruses modulate IFN I, demonstrating the importance of the V proteins in blocking IFN induction and signalling (Horvath, 2004). These proteins are expressed following RNA editing of the mRNA that encodes P protein. Due to the lack of reagents for the avian IFN I system, this work has barely extended to avian paramyxoviruses. The likely role of the NDV V protein has, however, been demonstrated using genetically modified viruses (Park *et al.*, 2003a). Thus mutant viruses, defective for V protein expression (V^- NDV), replicate poorly in embryonated eggs and chicken embryo fibroblasts. This defect can be complemented by transfection of a plasmid expressing cDNA encoding the V protein into V^- NDV-infected cells (Park *et al.*, 2003b) or by insertion of the influenza virus NS1 gene into the V^- NDV. The NS1 gene allows the modified NDV to replicate better in human cells than does the parental NDV, suggesting the NDV V protein is more effective in modulating the chicken IFN system than the mammalian IFN system (unlike influenza NS1 which modulates both systems effectively). These differences are likely to be important for the host range specificities of the two viruses. IFN-sensitive NDV can also be rescued by transfection of a plasmid expressing cDNA encoding NDV V protein (Park *et al.*, 2003b). Although all examined V proteins of mammalian paramyxoviruses appear to be involved in modulating the mammalian IFN response, the actual detailed mechanism by which they do so varies from virus to virus. Elucidation of the mode of action of the NDV V protein will, therefore, require the availability of a full panoply of reagents specific for the avian IFN I system.

Immunoevasion Mechanism of the Avian Reoviruses

It is clear that dsRNA is a powerful inducer of the antiviral type I IFN system. Although the positive-strand RNA viruses, and even poxviruses, generate some dsRNA during their replication and transcription, the dsRNA viruses must be able to prevent recognition of their dsRNA genome by the cell. This is probably largely achieved by ensuring that the genome is never exposed outside of the capsid within the cytoplasm. However, the avian reoviruses, in common with mammalian reoviruses, appear to encode a dsRNA-binding protein σA that can mask dsRNA from cellular dsRNA binding proteins such as the dsRNA-dependent protein kinase PKR. Thus σA functions like poxvirus E3, and can actually replace E3 in Vaccinia virus (Gonzalez-Lopez *et al*., 2003).

CONCLUSIONS

Immunosuppression as a consequence of (subclinical) virus infections is a common occurrence with important consequences for the poultry industry. Thus far, most studies describe the effects on immune responses without addressing the mechanistic aspects of the immunosuppression, which is the consequence of the lack of reagents and appropriate techniques. However, during the last few years, and especially since the chicken genome has been sequenced, research has started to focus on the interactions between virus infection and the effects on the (de)regulation of the immune responses. It is expected that rapid progress will be made in the next 5–10 years in this area of research.

There has also been a burgeoning interest in the mechanisms by which viruses evade or modulate the innate resistance mechanisms of the host, in particular the IFN-I system. This interest has so far had little impact on the study of avian viruses because of the lack of essential reagents. The recent derivation of the draft chicken genome sequence has opened up easier access to such reagents and it is likely that the coming years will see a rapid increase in the number of studies, and hence our knowledge of the intricacies of avian virus-host systems. Recent developments allowing manipulation of the large and complicated genome of IBV (Casais *et al*., 2001), a coronavirus like the SARS virus, may also help identify additional viral immunomodulators, and hopefully elucidate their mode of action (Casais *et al*., 2005; Hodgson *et al*., 2006). Such technology has been available for some time for avian adenoviruses but the divergence between mammalian and avian adenoviruses has made it difficult to predict candidate immunomodulators. Hopefully reannotation of the genome, which identified three putative surface glycoproteins with immunoglobulin-like folds, will be useful for further analysis (Washietl and Eisenhaber, 2003). That this type of study is likely to elucidate new paradigms, which may even be relevant beyond the avian host, is illustrated by the relative dearth in avian herpesviruses and poxviruses of obvious immunomodulators of the type observed in their mammalian cousins. The emergence of West Nile virus and of H5N1 AIV as threats to wild birds, farmed poultry, mammalian livestock and companion animals and to humans, may lead to provision of resources needed to understand the complexity of the interactions of these and other viruses with their avian hosts. Hopefully as a consequence, we will be better equipped to control and treat, or possibly even eradicate, such viruses.

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17 FACTORS MODULATING THE AVIAN IMMUNE SYSTEM

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INTRODUCTORY STATEMENT

There are many factors that influence the type and intensity of immune responses to pathogens. These include intrinsic factors such as age and sex of the individual, as well as extrinsic factors such as environmental conditions, social interactions, exposure to toxicants and the type of diet. This chapter explores the non-genetic factors that impact the immune response of birds as well as the underlying endocrine basis of many of these changes. Finally, methods for assessing the changes in immunity that result from non-genetic influences are discussed for both poultry and free-living birds.

ENDOCRINE REGULATION OF IMMUNITY

There are interactions between metabolic, immunological and neuroendocrine systems that affect animal physiology and homeostasis. Stress hormones, metabolic hormones, sex hormones and other endocrine signalling molecules can directly or indirectly affect the immune system. These compounds mediate many of the changes in immunocompetence caused by diet, stress and life history events such as development, growth, reproduction, migration and moulting.

Stress Hormones: Epinephrine, Norepinephrine, Dopamine and Glucocorticoids

The "stress" hormones are generally associated with acute or chronic stressors that alter an animal's homeostasis. Acute stress results in a coordinated effort to remove the animal from the stressful environment and involves the "fight-or-flight" response driven by catecholamines such

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as epinephrine, norepinephrine and dopamine, which increase cardiovascular function (reviewed by Dohms and Metz, 1991), heart rate and muscle blood flow (reviewed by Ligier and Sternberg, 1999). Simultaneously, hypothalamic corticotrophin-releasing factor (CRF) and vasopressin secretion stimulate release of adrenocorticotropic hormone (ACTH), and resulting glucocorticoid secretion from the adrenal cortex (Dohms and Metz, 1991). These compounds can exert direct effects on the immune system; catecholamines are released directly into lymphoid tissue as a result of direct innervation by sympathetic and parasympathetic nervous systems, and lymphocytes have high-affinity receptors for ACTH and glucocorticoids that are up regulated by immune stimulation (reviewed by Mumma *et al*., 2006). Lymphocytes can also regulate the stress response since they can synthesize ACTH, corticosterone and other neuroendocrine mediators (Smith *et al*., 1986; Mashaly *et al*., 1993).

In addition to immune responses, stress hormones and predominantly glucocorticoid, affect metabolism, activity levels, cognitive and learning processes, reproductive behaviour, parent–offspring interactions (reviewed by Rubolini *et al*., 2005), as well courtship, copulation and hunting (reviewed by Mostl and Palme, 2002). Glucocorticoid levels vary with an individual's social status; in cooperative breeding systems, dominant individuals generally have higher glucocorticoid levels compared to subordinates. However, in winter flocks of birds, subordinates often have higher glucocorticoid levels, presumably due to competition for scarce food resources (reviewed by Lindstrom *et al*., 2005). Thus, when examining effects of stress hormones on immunity, a variety of factors must be taken into consideration.

In general, research suggests that ACTH and glucocorticoid are immunosuppressive. In chickens, ACTH implants reduce humoral immunity and cutaneous basophil hypersensitivity (CBH) to phytohaemagglutinin (PHA) (Mumma *et al*., 2006). Additionally, induction of the ACTH/glucocorticoid axis generally reduces lymphocyte numbers (predominately B and natural killer (NK) cells) and increases heterophil numbers (Dhabhar *et al*., 1995, 1996). Glucocorticoids, predominantly corticosterone, induce rapid apoptosis in immature T- and B-cells (reviewed by Lechner *et al*., 2001). However, it is clear that the level of corticosterone, the time of exposure (e.g. short-term or long-term exposure) and the type of immune parameter measured affect corticosterone responses (reviewed by Martin *et al*., 2005). In general, initial corticosterone increase allows for a more robust immune response to challenge, while chronically increased corticosterone results in immunosuppression. Initial corticosterone elevation stimulates CBH function (Dhabhar and McEwen, 1997; Martin *et al*., 2005), alters skeletal protein metabolism (Klasing *et al*., 1987), and carotenoid partitioning in growing birds (Table 17.1), shifts the adaptive immune response from a Th1 to a Th2 type of response (reviewed by Ligier and Sternberg, 1999) and reduces whole blood lymphocyte proliferation (Table 17.2). In contrast, chronic elevation of corticosterone acts in a negative feedback manner to regulate immunity and suppress immune responses (Dhabhar and McEwen, 1997; Mashaly *et al*., 1998). Chronic corticosterone administration reduces bursal, thymic and splenic mass (Dohms and Metz, 1991), increases NK cell function, suppresses primary and secondary antibody responses, suppresses phagocytosis and prostaglandin E2 (PGE_2) production by adherent peripheral blood

TABLE 17.1 Effect of Short-Term Noise Stress on Tissue Carotenoid Concentration

	Thymus Lutein + Zeaxanthin (μmol/kg)	Bursa Lutein + Zeaxanthin (μmol/kg)	Liver Lutein + Zeaxanthin (μmol/kg)
6 h time point	1.85 ± 0.15^{a}	0.21 ± 0.08	0.52 ± 0.09^{a}
24 h time point	0.58 ± 0.15^{b}	0.22 ± 0.06	0.30 ± 0.13^{b}

Four-week old laying hen chicks (n = 8/treatment) were exposed to noise created by moving and sampling of chicks not involved in the experiment for 6 or 24 h, at which time tissue carotenoid levels were measured using standard methods.

[a–b]Within a column, means with different superscripts are significantly different from the 6 h time point ($p < 0.05$) (T.V. Leshchinsky, E.A. Koutsos, and K.C. Klasing, unpublished observations).

TABLE 17.2 Effect of Short-Term Noise Stress on *Ex Vivo* Lymphocyte Proliferation

	Proliferation Index, Mean ± SEM	
	PHA-P	**conA**
Non-stressed birds	14.96 ± 2.29[a]	16.01 ± 2.59[a]
Stressed birds	8.58 ± 1.10[b]	8.01 ± 0.70[b]

Four-week old broiler chicks (n = 12/treatment) were either non-stressed or stressed (noise created by moving and sampling of chicks not involved in the experiment) for 2 h. Lymphocyte proliferation to phytohaemagglutinin-P (PHA-P) (25 μg/ml) or concanavalin A (conA) (100 μg/ml) was measured in whole blood using standard methods.

[a–b]Within a column, means with different superscripts are significantly different ($p < 0.05$) (T.V. Leshchinsky, E.A. Koutsos and K.C. Klasing, unpublished observations).

monocytic cells (Fowles *et al*., 1993) and reduces lymphocyte proliferation, as well as interleukin (IL)-2 and interferon (IFN)-γ production (Isobe and Lillehoj, 1992).

Effects of stress hormones are clearly detectable in an animal experiencing a stressor, but it is important to note that in reproductively active females, stress responses are correlated to the level of deposition of corticosterone into the egg (Hayward and Wingfield, 2004), resulting in the effects of stressors on the hen affecting the development and stress responses of the progeny. Chicks hatched from eggs with elevated corticosterone have slower growth rates and increased adrenocortical responses to acute stress (reviewed by Rubolini *et al*., 2005). Similarly, inoculation of corticosterone into gull eggs resulted in longer incubation times, lower rates of begging by chicks and reduced CBH responses (Rubolini *et al*., 2005). Interestingly, the effect of corticosterone on chick physiology appears to be dependent on sex hormones; male chicks with elevated egg corticosterone have reduced growth rates, while females have reduced corticosterone responsiveness after hatching (Hayward *et al*., 2006).

Other neuropeptides and neurotransmitters are implicated in the stress response, including serotonin, vasoactive intestinal peptide (VIP), vasopressin and oxytocin, acetylcholine and somatostatin (reviewed by Dohms and Metz, 1991). Several gastrointestinal-derived compounds, including cholecystokinin, substance P, somatostatin and VIP, generally increase gastrointestinal immune parameters, including mucosal lymphocyte proliferation and antibody production, monocyte chemotaxis and intraepithelial NK cell activity (reviewed by Bienenstock *et al*., 1989). The basis for regulatory effects is related to direct actions on leukocytes because receptors for these compounds are present on leukocytes (Bienenstock *et al*., 1989).

Sex Hormones: Testosterone, Oestradiol and Other Androgens

Sex hormones also affect immune responses of birds but the data are often conflicting. The basis for variable effects of sex hormones may be due to indirect effects of these hormones, differences in natural history of the species in question (e.g. age, breeding system) and environmental effects (e.g. free-living versus captive species). The natural history of the species may also affect the sex hormone response, since the magnitude and duration of sex hormone production is variable due to factors such as mating system and clutch size. For example, males in polygynous mating systems, as opposed to monogamous species, have higher levels of testosterone throughout the breeding season. Parental effort alone has been linked to reduced immune status in a variety of species (reviewed by Casto *et al*., 2001).

Sex hormones may directly affect immune responses; androgen receptors are located in the bursa of immature chickens (reviewed by Owen-Ashley *et al*., 2004), and development of thymus and bursa are influenced by steroid hormone levels (Glick, 1984). Oestrogen enhances the

phagocytic and cytostatic function of macrophages, but decreases chemoattractant expression (reviewed by Salem *et al*., 2000). In mammals, β-oestradiol reduces delayed-type hypersensitivity (DTH) responses and production of IL-2 and IFN-γ, while increasing levels of IL-4 and IL-10 (Salem *et al*., 2000), which suggests that oestrogen can shift the immune system from a Th1 to a Th2 type of response (reviewed by Erbach and Bahr, 1991).

Testosterone also affects immune responses, although results vary depending on study design. Many studies have used testosterone implants to experimentally increase testosterone levels, but effects of these implants are often contradictory to the effects of testosterone in non-implanted animals. For example, testosterone implants depressed humoral immunity in superb fairy wrens (*Malurus cyaneus*), but testosterone levels in non-implanted males were positively correlated with humoral immunity (Peters, 2000). Testosterone implants may affect immunity by a different mechanism than endogenous testosterone, since implants increase corticosterone levels (Owen-Ashley *et al*., 2004). Differences in environment may also mediate effects of testosterone implants. Free-living males with testosterone implants had stronger suppression of the CBH response than did implanted captive males, which could be related to differences in energy expenditure, food availability and other natural behaviours (Casto *et al*., 2001). In contrast to the effects of testosterone implants, other androgen implants have not shown induction of corticosterone responses.

The direct effects of testosterone are generally associated with immunosuppression since testosterone treatment causes premature bursa atrophy, suppresses antibody responses in chickens (Glick, 1984), and induces eosinophilia associated with increased parasite loads in barn swallows (*Hirundo rustica*) (Saino *et al*., 1995). In red jungle fowl (*Gallus gallus*), testosterone treatment reduces lymphocyte numbers and in starlings (*Sturnus vulgaris*) it decreases indices of cell-mediated and/or humoral immunity (Duffy *et al*., 2000). Supplemental testosterone reduces CBH responses and secondary antibody responses in some, but not all, species (Owen-Ashley *et al*., 2004). In addition to effects of testosterone and oestrogen, other androgens may affect avian immunity. Dehydroepiandrosterone (DHE), an androgen precursor, is the only androgen elevated in the blood of male song sparrows (*Melospiza melodia*) during the non-breeding season, and may be immunoenhancing based on *in vitro* studies (Owen-Ashley *et al*., 2004).

As with corticosterone, egg androgen concentrations affect the physiology of progeny. Yolk androgens positively affect embryonic and postnatal rates of development, competition between offspring (reviewed by Muller *et al*., 2005), as well as hatchling dominance and survival (reviewed by Hayward and Wingfield, 2004). However, it is hypothesized that high levels of yolk androgens are detrimental to offspring, since the associated accelerated growth may increase vulnerability to starvation, increase oxidative stress, and may result in a trade-off between skeletal growth and development of the immune system (Demas, 2004).

Metabolic Hormones: Thyroid Hormone, Insulin, Growth Hormone and Leptin

Thyroid hormone is a primary mediator of basal metabolic rate in animals, but also affects immune function via direct action on leukocytes, which have nuclear receptors for thyroid hormones (reviewed by Bachman and Mashaly, 1987), and/or stimulation of thyroid hormone synthesis in mammals (Bagriacik *et al*., 2001). Thyroid hormone positively affects thymus growth in birds (reviewed by Glick, 1984), affects humoral immunity and the number of circulating lymphocytes in birds, and is positively correlated to IL-2-like activity in chickens (reviewed by Bachman and Mashaly, 1987). However, thyroid hormone responsiveness differs between species and/or age and may be more effective during growing stages (Fowles *et al*., 1997).

Insulin and growth hormone, in mammals, act directly on leukocytes that have receptors for these hormones (reviewed by Griffin, 1989). Additionally, growth hormone mRNA has been identified in the thymus, spleen and bursa of chickens, and its concentration and composition is age- and tissue-specific (Luna *et al*., 2005). Effects of insulin and growth hormone include enhanced lymphocyte activation and counteraction of the inhibitory effects of corticosterone (Griffin, 1989). Additionally, growth hormone can influence thymic growth and maturation of T-lymphocytes (Johnson *et al*., 1993).

Leptin, a peptide hormone synthesized and secreted by adipose tissue, is also implicated in immunomodulation. Leptin deficiency leads to lymphoid atrophy and reductions in lymphocyte number, and leptin appears to have direct effects on the central nervous system and neurotransmitter production (reviewed by Demas, 2004).

Environmentally Responsive Hormones: Melatonin

The avian pineal gland synthesizes and secretes melatonin, which provides information to the brain on day length, or photoperiod, and coordinates reproductive function in many species (reviewed by Nelson and Demas, 1997). Avian melatonin differs from the mammalian counterpart. As in other lower vertebrates, avian pinealocytes have direct photosensitivity, which is lacking in mammalian pinealocytes. As a result, light inhibits melatonin synthesis in birds whereas darkness stimulates melatonin synthesis in mammals. Avian pinealocytes also have persistent melatonin rhythmic synthesis in constant darkness, which mammals do not have (reviewed by Skwarlo-Sonta, 1999). This rhythmic synthesis leads to variation in melatonin binding capacity in thymus and bursa during light (higher) and dark (lower) periods, but not in spleen or other tissues. Data suggest that either binding sites are downregulated in immune tissues in response to diurnal patterns or several types of melatonin receptors exist (reviewed by Poon *et al.*, 1994). The mechanism by which melatonin affects immune responses is not yet clear, although effects on opioid peptides and cytokines like IFN-γ and IL-2 have been suggested (Poon *et al.*, 1994), as well as effects on Ca^{2+} channels and intracellular Ca^{2+} concentration (Skwarlo-Sonta, 1999). Finally, melatonin may be part of an integrated system involving glucocorticoids. In mammalian species, melatonin treatment can ameliorate effects of glucocorticoids (Nelson and Demas, 1997).

Melatonin receptors are present on avian spleen, thymus and bursa (reviewed by Skwarlo-Sonta, 1999), and are presumably present on lymphocytes, since melatonin stimulates cytokine production by lymphocytes (Poon *et al.*, 1994). Pinealectomy during embryogenesis reduces thymic and bursal growth and cell numbers, and reduces parameters of humoral and cell-mediated immunity (Skwarlo-Sonta, 1999). Similarly, accelerated development of cellular and humoral immune responses was observed when newly hatched turkey poults were administered melatonin, or when melatonin was administered *in ovo* just prior to hatching (Moore and Siopes, 2005). Later in life, melatonin levels and binding decrease (Poon *et al.*, 1994). Thus, it appears that melatonin enhances parameters of the immune system, albeit in coordination with other hormonal components, and effects are most noticeable early in life.

PHYSIOLOGICAL STATES

Social and environmental stresses are common in the life of birds and influence the immune system and susceptibility to diseases. Changes in immunity are thought to be orchestrated primarily by hormones, although direct neural modulation and nutritional changes may also be involved. The diet itself is also a major determinant of the type and/or magnitude of immune response. Although nutrient deficiencies receive the most attention, there are many nutrients that modulate immunity even at levels between the dietary requirement and toxic levels. In fact, many nutrients that are not normally considered to be either required or toxic are immunomodulatory.

Stress: Environmental, Social, Noise, Air

Stress, in this context, will be defined as per Dohms and Metz (1991), as an adaptive response to threats to an animal's homeostasis. Stressors may be external or internal stimuli, and the severity, duration, novelty and host status will all affect the response to the stressor. Many environmental stressors affect immune responses, and this topic has been reviewed extensively

(Dietert *et al*., 1994). Potential stressors include temperature, light (e.g. ultraviolet light), air quality (e.g. ammonia, ozone), infectious agents, environmental contaminants (e.g. mycotoxins, pesticides) and nutrients.

Temperature Stressors

Exposure of birds to extreme temperatures can impact immune responses and the effect of heat or cold stress is generally immunosuppressive (reviewed by Dohms and Metz, 1991), particularly in terms of CBH responses and lymphocyte proliferation (*ex vivo*), with variable effects on humoral responses (Regnier and Kelley, 1981). The mechanism by which heat stress modulates immunity is in part due to the extent of induction of heat shock proteins in lymphocytes, heterophils and macrophages (Dietert *et al*., 1994), while cold stress suppresses plasma corticosterone levels and enhances thyroid hormone levels (Hangalapura *et al*., 2004).

Other Environmental Stressors

The physical environment can affect the stress response and thus the immune response. For example, chickens raised on slatted floors have elevated heterophil:lymphocyte ratios and duration of tonic immobility, and reduced antibody titres and CBH responses as compared with birds in a litter-based environment (El-Lethey *et al*., 2003). Elevated stocking density is negatively correlated to the growth of immune organs (Heckert *et al*., 2002). Similarly, increasing breeding density of wild birds reduces CBH responses, although this is also correlated to body size and presumably food availability (Tella *et al*., 2001). In laying hens, providing access to perches and the ability to engage in natural behaviours decreases heterophil:lymphocyte ratios (Campo *et al*., 2005).

The geographical environment in which a bird lives also affects immune responses, thus the same species may have different responses depending on geographical location. For example, corticosterone levels were elevated to a much greater degree in house sparrows (*Passer domesticus*) from temperate environments than from tropical environments (Martin *et al*., 2005). These effects may be due to environment (e.g. photoperiod, which can affect corticosterone and immune function) or they may be part of differing evolutionary selection pressures in different environments. Additionally, it has been proposed that birds living in tropical environments have increased parasite loads, and thus mount lower corticosterone responses in order to maintain adequate immune defences (Martin *et al*., 2005). Alternately, birds living in temperate environments may mount corticosterone responses when challenged and when other resources (e.g. nutritional resources) are available to allow this response to occur.

Dietary Effects on Immunity

Nutrition is an important regulator of the immune system. An appropriate balance and quantity of nutrients are important for the development, maintenance and response of the immune system. There is little doubt that severe nutritional deficiencies impair the immune system and increase susceptibility to infectious diseases. The immune system is sensitive to even moderate deficiencies of some nutrients. However, the immune system is essential for life and appears to have a very high priority for many nutrients relative to muscle accretion or reproduction and is surprisingly resilient to severe deficiencies of some nutrients. Understanding which nutrient falls into each of these categories is of great practical importance. Additionally, many nutrients that are not essential for growth or reproduction regulate and modify immune responses. Modulation of the immune system by diet has been employed for two primary goals in poultry: to decrease the incidence of infectious diseases, and to minimize the untoward effects of immune responses on growth, egg production and the incidence of metabolic diseases (Cook *et al*., 1993).

Nutritionally Critical Nutrients

Deficiencies of many of the essential nutrients that are sufficiently severe to slow growth or reproduction are also deleterious to the immune system (for reviews, see Cook, 1991; Latshaw, 1991;

Klasing, 1998b; Kidd, 2004). For some nutrients, the immune system is among the most sensitive of any tissue to moderate deficiencies, while for other nutrients the immune system is unaffected by deficiencies. Two mechanisms appear to mediate this dichotomy. First, leukocytes have an excellent capacity to compete with other tissues for low levels of some, but not all, nutrients. Second, an immune response is accompanied by the mobilization of nutrients from muscle and other tissues, which supplies adequate amounts of some, but not all, nutrients to leukocytes (Klasing, 1998a).

Leukocytes express levels or types of proteins that facilitate the accumulation of nutrients and endow them with a high priority relative to other tissues. For example, chicken macrophages express very high levels of metallothionein when activated and this permits them to accumulate high levels of zinc (Laurin *et al*., 1990) to supply their anabolic needs. This property endows the macrophage with a higher priority for limiting levels of this trace nutrient relative to many other cell types. This is important because the need for trace nutrients markedly increases when cells are activated and become highly anabolic. A similar situation exists for the essential amino acid lysine; bursal cells express high levels of high-affinity lysine transporters relative to skeletal muscle and are protected during periods of dietary lysine deficiency (Humphrey *et al*., 2006). It appears that evolution has endowed some leukocytes with a high priority for at least some nutrients; likely those that were commonly deficient in natural diets as animals evolved. Nutrients that appear to fall into this category include energy supplying compounds such as glucose (Humphrey *et al*., 2004), zinc (Mohanna and Nys, 1999) and most of the amino acids (Kidd, 2004) except possibly methionine (Cook, 1991; Rama Rao *et al*., 2003). Conversely, a lack of accumulation systems that establish priority among cell types prevents prioritization of some nutrients to leukocytes and they are affected during deficiencies in a manner similar to all other tissues (e.g. copper) (Koh *et al*., 1996).

During a pathogen challenge or following vaccination, the acute phase response diverts nutrients from skeletal muscle and other tissues. These nutrients supply the increased demand of responding leukocytes and liver for the production of acute phase proteins. Although the nutrient needs for leukocytes are relatively small relative to those used for growth or reproduction, the nutrients needed for protection of epithelial surfaces and for hepatic production of acute phase proteins is sizable (Klasing, 2004). In the case of copper, which is a cofactor for several acute phase proteins, the requirement of broiler chicks clearly increases following robust immune responses and higher dietary levels are useful (Koh *et al*., 1996). But in the case of lysine, the increased need for protective processes is compensated by a slower growth rate orchestrated by the immune response and higher dietary levels are not helpful in mitigating the growth depression (Klasing and Barnes, 1988). Much work remains to be done to define the exact changes in nutrient requirements that accompany robust immune responses, but clearly the appropriate balance of dietary nutrient changes with the health status of the animal.

In general, developing T-lymphocytes are the most susceptible leukocyte population to nutrient deficiencies. Severe deficiencies of amino acids like lysine (Humphrey *et al*., 2006), or branched-chain amino acids (leucine, isoleucine, valine) that markedly impair growth of chicks, cause involution of the thymus but not the bursa (Konashi *et al*., 2000). The thymus is more sensitive to deficiencies of the branched-chain amino acids than any other group of amino acids. However, the deficiency must be severe and the thymus and peripheral T-cell populations are not sensitive to marginal deficiencies of the branched-chain amino acids (Konashi *et al*., 2000). With diminished lymphocyte-mediated immunity, exaggerated responses by the innate immune system often occur during infection, which result in robust inflammatory responses and accompanying immunopathology.

Feed restriction is commonly used in the management of poultry. A wide variety of studies have examined the influence of feed restriction on immunocompetence (Nir *et al*., 1996; Praharaj *et al*., 1996; Hangalapura *et al*., 2005) and the general conclusion is that mild to moderate feed restriction enhances many measures of immunity. Very severe feed restriction impairs cellular immunity but may enhance innate (Hangalapura *et al*., 2005) or antibody responses (Khajavi *et al*., 2003). The endocrine changes that accompany feed restriction likely mediate many of the effects on immunocompetence (Nir *et al*., 1996).

Immunoregulatory Nutrients

Some essential nutrients, when fed at dietary levels that are clearly above the nutritional requirement, modulate the immune system. Additionally, some nutrients that are not normally considered as being dietary essential may also modulate immunity. Nutrients that have strong immunomodulation activities include long-chain polyunsaturated fatty acids (PUFA), carotenoids, isoflavonoids (e.g. genistein) and vitamins A, C, D and E (Cook, 1991; Latshaw, 1991; Cook *et al*., 1993; Dietert *et al*., 1994; Klasing, 1998b; Kidd, 2004). The profile of immunomodulatory properties of these nutrients in chickens are summarized in Table 17.3. Unlike increases in nutrients from deficient to sufficient levels, where most indices of immunocompetence are elevated, supplementation of immunomodulatory nutrients causes some components of immunity to be elevated and others to be diminished; in other words, the type and intensities of responses have been changed or modulated.

Many immunoregulatory nutrients influence the balance of cytokines and eicosanoids released by regulatory cells. For example, several nutrients change the activity of nuclear factor-kappa B (NFκB) and, consequently, modulate cytokine expression. Nutrients that decrease NFκB activity and dampen the inflammatory response include a variety of fatty acids, antioxidants, vitamin A and lutein. In addition to dampening the inflammatory response by decreasing the release of pro-inflammatory cytokines, these nutrients often shift lymphocyte responses from T-cytotoxic (cell-mediated) responses towards antibody responses. These changes have implications for the susceptibility of experimental animals to authentic infections (Klasing and Leshchinsky, 1999). The anti-inflammatory properties of PUFA in the n-3 series, conjugated linoleic acid, lutein and xylitol may be useful in mitigating the growth depression that accompanies inflammatory responses (Table 17.3). However, because of their immunomodulatory effects on the Th1:Th2 balance, the benefits of these nutrients are dependent on the pathogens present in the environment.

TABLE 17.3 Immunomodulatory Nutrients and Their Effects on Innate and Adaptive Responses

Nutrient	Change in Innate Response	Change in Adaptive Responses	References
Vitamin E	Dampened acute phase response; increased macrophage phagocytosis	Increased antibody response	Swain *et al*. (2000), Leshchinsky and Klasing (2001), Sijben *et al*. (2002), Konjufca *et al*. (2004), Lin and Chang (2006)
Long-chain n-3 fatty acids	Dampened acute phase response	Dampened cell-mediated responses; increased antibody responses	Fritsche and Cassity (1992), Korver and Klasing (1997), Korver *et al*. (1998), Sijben *et al*. (2001), Parmentier *et al*. (2002), Puthpongsiriporn and Scheideler (2005)
Vitamin A		Increased antibody responses	Friedman and Sklan (1989), Sklan *et al*. (1994), Lessard *et al*. (1997)
Lutein	Dampened acute phase response; increased macrophage NO release	Increased mitogen responses of T-lymphocytes	Koutsos *et al*. (2006), Selvaraj *et al*. (2006)
Xylitol	Dampened acute phase response	Increased antibody response and mitogen responses of T-lymphocytes	Takahashi *et al*. (2000), Takahashi and Akiba (2005)
Conjugated linolenic acid	Dampened acute phase response and superoxide from macrophages; increased phagocytosis	Increased antibody response	Miller *et al*. (1994), Takahashi *et al*. (2002, 2003), Politis *et al*. (2003), Zhang *et al*. (2005)

Immunomodulatory nutrients are defined as those nutrients that rebalance the immune response and act at levels well above the established NRC (2005) requirements for growth and productivity. Studies that examined the effect of correcting a nutrient deficiency are not considered in this summary.

The raw ingredients of grain–soybean diets used to feed poultry and livestock contain levels of several immunomodulatory nutrients that set the background for dietary intervention. Corn contains fat that is rich in the n-6 fatty acid, linoleate and several carotenoids, whereas soybean meal contains isoflavonoids like genistein. Both of these ingredients are low in vitamins E and A. These main ingredients are usually supplemented with additional fat, amino acids, vitamins, minerals and carotenoids, many of which provide additional immunomodulation activity. The immunomodulatory nutrients appear to have robust dose–response profiles and these should be taken into consideration when determining the amount to supplement. For example, several investigators have titrated the dose–response curve for vitamin E (Friedman *et al.*, 1998; Leshchinsky and Klasing, 2001; Lin and Chang, 2006). The National Research Council (NRC, 2005) identified the requirement for vitamin E as 10 IU/kg diet. Moderately higher levels of vitamin E (25–50 IU/kg) enhance antibody titres following a vaccination but still higher levels (>150 IU/kg) are suppressive. These studies, as well as those examining vitamin A (Sklan *et al.*, 1995), illustrate that the immunomodulatory actions of nutrients often follow a bell-shaped curve and that high levels may not be as useful as moderate levels.

Unfortunately, the net immunomodulatory influences of a diet are not a simple sum of the actions of individual nutrients because there are robust interactions between different immunomodulatory nutrients. For example, dietary fatty acids of the n-3 and the n-6 series have separate and interactive effects when supplemented to diets of chicks (Sijben *et al.*, 2001; Parmentier *et al.*, 2002). Similarly, the anti-inflammatory effect of dietary fatty acids on chicken macrophages depends on the amount of lutein in the diet (Selvaraj and Klasing, 2006; Selvaraj *et al.*, 2006). The opposite is also true; the anti-inflammatory effects of lutein are dependent on the amount of n-3 fatty acids in the diet. This interaction is mediated by nuclear hormone receptors that respond to these two nutrients (RXR and PPAR, respectively), which in turn affect the expression of NFκB. Both experimental and clinical results clearly indicate that the specific immunomodulatory actions of a nutrient and its interactions with other dietary nutrients must be understood before application to avian populations because its efficacy is context dependent. The resulting changes may be protective for some pathogens, i.e. those that are controlled by the elevated immune responses. However, the resulting changes may increase susceptibility to those pathogens that are controlled by the diminished responses (Klasing and Leshchinsky, 1999).

TOXIC SUBSTANCES

Birds are exposed to many potential toxins that may influence the immune system. In birds, various mycotoxins, pesticides, organochlorine compounds, petroleum hydrocarbons, minerals, organometallics and radiation have been evaluated for their immunotoxic potential (reviewed by Dietert *et al.*, 1994; Fairbrother *et al.*, 2004). Among these, the mycotoxins are of greatest concern to the poultry industry because they are very common contaminants of grains (see below). Minerals that contaminate feedstuffs, supplements or water are also of concern because of their potential for toxicity (reviewed in NRC, 2005). Although the maximum tolerable levels set by NRC for each mineral are not typically based on immunotoxicity, changes in immune functions are among the most sensitive indices of toxicosis of several minerals (e.g. lead and mercury). The developing immune system is often more sensitive to toxic substances than the mature immune system (e.g. lead) (Lee and Dietert, 2003).

Mycotoxins

Mycotoxins are toxic secondary metabolites produced by filamentous fungi, possibly to limit competition with other fungal species, although many theories about the function of their secondary metabolites exist. Ingestion of mycotoxins by animals may result in pathology, and often in immunosuppression. Mycotoxins can suppress immune responses due to hepatotoxicity (e.g. aflatoxin B_1), atrophy of immune organs during development (e.g. aflatoxin B_1, ochratoxin A,

T-2 toxin), suppression of cell-mediated immunity (e.g. aflatoxin B_1), nephrotoxicity (e.g. ochratoxin A), and result in increased susceptibility to infection (reviewed by Corrier, 1991; Dietert *et al*., 1994; Surai and Mezes, 2005). Mycotoxins also exert direct effects on the local gastrointestinal immune system, including reduction of epithelial integrity and intestinal barrier function (e.g. fumonisin B_1, ochratoxin A, deoxynivalenol and patulin), reduced enterocyte proliferation (e.g. aflatoxin B_1 and T-2 toxin in addition to those affecting barrier function), increased mucus synthesis (e.g. fumonisin B1), altered cytokine production (e.g. increased transforming growth factor (TGF)- β and IFN-γ by deoxynivalenol, reduced IL-8 by fumonisin B_1) and altered numbers of IgA-producing cells (e.g. increased by deoxynivalenol, reduced by T-2 toxin) (reviewed by Bouhet and Oswald, 2005). Mechanisms of mycotoxin-induced immunosuppression have been reviewed (e.g. Corrier, 1991; Dietert *et al*., 1994). It is important to note that the immunosuppressive effects of mycotoxins depend on dosage, route of administration, animal species, age and sex. Finally, more than 300 fungal secondary metabolites are recognized as toxic; of these perhaps 50 have been given some investigative attention. It is rare that a single mycotoxin is consumed in the diet – more often, several toxins are present. These toxins may work in an additive, synergistic or antagonistic manner, and much research remains to determine these interactions.

ASSESSMENT

Assessing changes in immunocompetence due to environmental factors such as stress, nutrition, toxicants or life history events (e.g. moulting, migration, reproduction) requires a systematic approach that yields results predicting "real world" protection against infectious and neoplastic diseases. Importantly, assessment should be able to distinguish between positive changes in immunocompetence and potential adverse ones, including immunosuppression, adverse immunostimulation, immunogenicity, hypersensitivity and autoimmunity. A standardized panel of assays for assessing immunosuppression due to toxicants has been developed (Luster *et al*., 2003; Germolec *et al*., 2004a, b). However, this panel has not been tested for relevance to more physiological forms of immunomodulation, such as those due to stress, nutritional variation or life history events. Extensive research in immunotoxicology indicates that a broad array of highly quantitative assays is necessary to predict the effectiveness of the immune system to thwart infectious and neoplastic diseases and that there is no single assay of an immune function that is sufficient. The immunotox panel includes general measures of health (body weight, food intake and differential leukocyte counts), the weight and histopathology of primary and secondary immune organs, and functional assays of immune responses to antigens (Center for Drug Evaluation and Research, 2002). Functional assays that integrate across the cellular and regulatory processes responsible for the expression of the major arms of immunity are initially most informative. Once a general survey of the immune system has been obtained, directed studies to unravel the responsible specific mechanisms are illuminating. The use of reference compounds (e.g. azathioprine, dexamethasone, cyclophosphamide and cyclosporin A) or nutrients is also important for interassay, interlaboratory and interspecies comparisons (Dean *et al*., 1998; Dean, 2004).

Studies designed to examine broad environmental effects on immunocompetence, including stress and diet, should include measures of both constitutive and inducible immunity afforded by the innate and adaptive arms of the immune system. *In vivo* assays are initially most appropriate because shifts in immune function are often due to changes in the hormonal, nutritional and physical milieu to which leukocytes are exposed. Additionally, dose–response curves are crucial because the effects of nutrients, toxicants and hormones are not linear and often biphasic (Leshchinsky and Klasing, 2001; Calabrese, 2005).

For *in vivo* tests of antibody responses, benign antigens such as keyhole limpet haemocyanin or sheep red blood cells or killed pathogens allow measurement of immune responses independently of an ongoing infection. The use of live attenuated vaccines to probe immune defences is fraught with problems of interpretation. A large response could be due to a better capacity to respond to the challenge dose. It also could be due to sufficiently poor immunocompetence of the animal, such that the challenge dose replicates rapidly to provide a larger

and persistent challenge, requiring a more sustained and vigorous response. In depth studies on rodents exposed to toxic chemicals indicate that T-cell-dependent antigens give better results in initial screening of immunocompetence than T-cell-independent antigens (Germolec, 2004; Holsapple *et al*., 2005).

In vivo tests of cell-mediated (Th1) responses are important and in immunotoxicology the DTH response to recall antigens has been adopted. In avian immunology the lectin, PHA, is sometimes used for this purpose by measuring the amount of swelling that it induces following injection into the wing web or toe web. However, use of PHA is fraught with problems of interpretation because the swelling is due to both inflammation and lymphocyte infiltration. Thus, it is not possible to disentangle innate and cell-mediated responses with this test.

Results from *in vivo* experiments can sometimes be further explored in *ex vivo* cultures of diluted whole blood (Millet *et al*., 2007). Whole blood retains much of the nutrient and hormonal milieu found *in vivo*, whereas purifying leukocytes followed by culture in a generic medium with foreign serum removes many important regulatory factors which often drive shifts in immunity. *Ex vivo* experiments are highly sensitive to changes in circulating stress hormones. For example, the immunosuppressive effects of only 30 min of acute stress are evident in the ability of whole blood to kill *Escherichia coli* (Millet *et al*., 2007).

Host resistance to challenges with infectious organisms or transplantable tumours is critical for final evaluation of immunomodulation. Unfortunately, this important step is rarely done because of the expense. When protection is quantified, the use of challenging organisms that probe Th1, Th2 and inflammatory immune responses should be utilized in order to distinguish immunosuppression from a shift in the emphasis of the response, i.e. immunomodulation. In immunotoxicology testing in rodents, compounds that cause moderate changes in indices of immunocompetence do not always translate into changes in resistance to authentic pathogens. This is likely because of the considerable redundancy and overlap of immune effector systems. Conversely, compounds producing no evidence of changes in functional tests of immunity are not likely to affect resistance to pathogens or tumours (Luster *et al*., 1992, 1993; Germolec, 2004).

Finally, the developmental stage affects the sensitivity of the immune system to perturbation. The developing immune system is more susceptible to toxins than that of the adult (Dietert *et al*., 1994; Holsapple *et al*., 2005; Luebke *et al*., 2006) and this situation is generally thought to be true for nutritional deficiencies, though little research has focused on this comparison in birds. For toxicants, exposure during development may have different qualitative effects (i.e. different immune parameters are affected) as well as quantitative effects (i.e. lowest effective dose and dose–response curve). Insults during development may also have more persistent effects, sometimes causing lifetime immunosuppression.

Special Consideration for Free-Living Birds

Studies in avian physiological ecology and evolution are defining an important new field in biology – ecoimmunology (see Chapter 22). Because the costs of different components of the immune system vary, a bias favouring some types of defences over others is postulated to reflect trade-offs between competing life history traits (Norris and Evans, 2000; Lee and Klasing, 2004). Studying immunity in free-living birds has many limitations due to the difficulties of catching and recapture of individuals, stress artefacts following capture, species specificity of reagents and the small size of most free-living birds.

Keeping free-living birds in captivity imposes many pressures such as stress and a lack of appreciation for diet and husbandry. Thus, it is desirable to sample birds immediately after capture and conduct assays on a single blood sample. Assuming blood contributes 6% of body weight, and that it is acceptable to remove 10% of the blood volume, only about 60 μl of blood is available for evaluation of immune function in a 10 g bird.

Most commercially available antibodies optimized for measuring chicken CD surface antigens, regulatory or effector peptides (complement, acute phase proteins) do not recognize the proteins of other species, or their binding affinities are sufficiently different to compromise

quantitative analysis. In summary, a panel of assays useful for free-living birds would be most useful if reagents are species independent, if it does not require holding animals in captivity, if it requires small blood samples, and if the results are quantitative and integrate across important cellular and regulatory processes that predict immune function. Such a panel would have the most utility if time-sensitive steps can be accomplished in field laboratories with limited infrastructure (see Chapter 22). Assays for microbial killing and phagocytosis activity of whole blood and plasma levels of natural antibodies, lysozyme, mannan-binding protein and complement-like lytic activity have been developed and validated for evaluating constitutive innate immunity in wild-caught birds (Matson *et al*., 2005, 2006; Millet *et al*., 2007). Unfortunately, currently there are no assays, for lymphocyte-mediated adaptive immune responses that satisfy these criteria.

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18 AUTOIMMUNE DISEASES OF POULTRY

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GENERAL CHARACTERISTICS OF AUTOIMMUNE DISEASES
AUTOIMMUNE VITILIGO IN SL CHICKENS
SPONTANEOUS AUTOIMMUNE (HASHIMOTO'S) THYROIDITIS IN OS CHICKENS
SCLERODERMA IN THE UCD-200 AND 206 LINES OF CHICKENS
REFERENCES

GENERAL CHARACTERISTICS OF AUTOIMMUNE DISEASES

The immune system has developed many effective ways to protect an individual from environmental insults and disease. Adaptive (specific) immunity will focus these defensive efforts very specifically on a given antigen in order to remove and/or destroy that antigen. However, when this specific response is directed against self-antigen, the result is autoimmune disease. Autoimmune disease is defined as a disease caused by a breakdown of self-tolerance such that the adaptive immune system responds to a self (autologous)-antigen and causes cell and tissue damage.

Autoimmune diseases can be categorized into two basic types: organ-specific and systemic diseases. In organ-specific or localized autoimmune diseases, immune recognition is cell-/tissue-specific as is the resulting immunopathology. Examples of organ-specific autoimmune diseases include Hashimoto's thyroiditis, type-1 diabetes, vitiligo and many others. In systemic autoimmune disease, such as systemic lupus erythematosus (SLE), multiple organs are affected. Although many of the autoimmune diseases are rare, according to the National Institutes of Health Autoimmune Diseases Coordinating Committee (2005) autoimmune diseases collectively affect 5–8% of the USA population. A disproportionate number of people with autoimmune diseases are women and, for unknown reasons, the prevalence of autoimmune disorders is increasing.

Self-tolerance is an essential property of the immune system designed to ensure that individuals do not respond to their own antigens. Tolerance to self-antigens is normally maintained by selective processes that prevent the maturation of self-antigen-specific lymphocytes and that inactivate or delete self-reactive lymphocytes that do mature and enter the periphery. Loss of self-tolerance may result from abnormal selection or regulation of self-reactive lymphocytes and by abnormalities in the way that self-antigens are processed and presented to the immune system.

Self-tolerance is primarily inherent in the T cell compartment. This is due in part to the rigorous selection processes T cells encounter during maturation in the thymus, the important regulatory function of T cells in adaptive immune responses and the restriction of T cells to recognition of antigenic-peptides in association with self major histocompatibility complex (MHC)

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molecules. Failures of self-tolerance within the T cell compartment can result in autoimmune diseases in which the autoimmune lesion is caused by cell-mediated and/or humoral immune responses.

Autoimmune diseases typically are multifactorial in nature, requiring several components such as genetic susceptibility, immunological influences and environmental factors for expression. Unfortunately, the relative contribution of these factors to the development of autoimmune disease is not clear cut and cannot be easily dissected. In organ-specific autoimmune diseases, genetic susceptibility is frequently associated with an inherent target cell defect that predisposes it to immunorecognition and may include aberrant immunological activity at various levels (e.g. dendritic cells/macrophages, B cells and T cells). The autoimmune destruction of cells has been found to be associated with a lack of regulatory function within the immune system, heightened immune activity and altered responsiveness of immune components to factors from other physiological systems. The role of environmental factors in the development of autoimmune disease is also multifaceted and may include infections by microbes as well as exposure to chemicals.

The consequences of having adaptive immune mechanisms specifically focused on a self-antigen are clearly destructive. It is puzzling, however, that autoimmune attacks against components such as the myelin sheath in multiple sclerosis, pigment cells in vitiligo and thyroid cells in autoimmune thyroiditis, do not necessarily destroy all of these target cells as would be implied by the specificity of the recognition. Rather, cell/tissue destruction tends to be progressive in nature, suggesting an important interplay between the target tissue and the antigens it displays, immune recognition and immunoregulation. Understanding these interrelationships will be important in the prevention and treatment of autoimmune disease.

Autoimmune disease does not appear to be of widespread concern in poultry production. Although, poultry breeding programmes have selected against inherited diseases, susceptibility to autoimmune diseases may be selected for in the absence of disease expression. Considering the frequently observed disorders associated with high intensity poultry production, such as leg, endocrine and nervous system problems, as well as metabolic, integumental, pulmonary, alimentary and reproductive tract problems, one may ask whether or not there is an underlying autoimmune component to these disorders. Given the multifactorial nature of autoimmune disease, the danger lies in the combination of factors encountered by a susceptible population that will lead to the expression of the disease. Hence, poultry breeding programmes will need to include attention to the role of the immune system in health and disease.

Over the years, the chicken has made significant contributions to the understanding of the components and mechanisms involved in autoimmune diseases, primarily because some lines of chickens spontaneously, and predictably, develop autoimmune disease. For example, the Smyth line (SL) chicken is the only animal model for the pigmentation disorder vitiligo, that manifests all the clinical and biological symptoms of the human disease (Plate 18.1(a)-(c)). The obese strain (OS) chicken (Plate 18.1(h)) is one of the most-valued and best models for spontaneously occurring Hashimoto thyroiditis and the University of California at Davis (UCD) 200/206 chicken lines are the only model for spontaneously occurring scleroderma that presents the combination of symptoms observed in humans (Plate 18.1(i) and (j)). Were it not for the highly observant and diligent efforts of geneticists like Drs J.R. Smyth Jr., R.K. Cole and P. Bernier, respectively, lines of chickens with these spontaneously occurring abnormalities would not have been developed. Their efforts have provided the biomedical community with valuable systems for research on the etiopathology, prevention and treatment of these and other autoimmune diseases. The study of autoimmune disease in susceptible lines of chickens has been a driving force for the development of assay systems, probes and tools to study fundamental aspects of immune function, immunopathology and immunophysiology in poultry. Moreover, the SL, OS and UCD-200/206 lines are excellent models to study the cause–effect relationship between a genetically controlled disease, immune function and environmental factors, and have been an incentive for the mapping of susceptibility genes and studies on the mechanistic links between susceptibility genes and failure of self-tolerance.

Reports on other occurrences of, and investigations into, autoimmune disease in poultry can be found in the literature. Included are experimental viral arthritis, experimental allergic encephalomyelitis and ovarian autoimmune disease (Blaszczyk *et al*., 1978; Pertile *et al*., 1996; Barua and Yoshimura, 2001). Due to the sporadic nature of these reports they will not be addressed in this chapter. Rather, the focus of this chapter will be the SL, OS and UCD-200/206 chicken models for autoimmune disease.

AUTOIMMUNE VITILIGO IN SL CHICKENS

Introduction

Vitiligo is a common dermatological disorder affecting at least 1% of the world's population. It is characterized by post-natal destruction of melanocytes in the skin generally resulting in patches of depigmentation and, in some individuals, complete depigmentation of the skin. There is a recognized association between autoimmune vitiligo and a variety of other autoimmune diseases, and individuals with vitiligo are more prone to develop skin cancer. In addition, the cosmetic disfiguration resulting from vitiligo leads to psychosocial effects that are particularly severe in the young and in people with dark skin pigmentation (Nordlund and Lerner, 1982; Spritz, 2006).

The mutant SL chicken is an important animal model for autoimmune vitiligo. Chickens from this line develop a spontaneous, vitiligo-like, post-hatch loss of melanocytes in feather and ocular tissue. The incidence of SL vitiligo (SLV) within a population of SL chickens typically ranges between 70% and 95%, with the majority of birds developing SLV between 6 and 12 weeks of age. Studies examining the basic defects manifested within the SL melanocyte described the presence of a competent pigment system at hatch (Plate 18.1(a)). Prior to visible signs of SLV, the earliest abnormality detected within SL melanocytes are irregularly shaped melanosomes containing pigmented membrane extension, hyperactive melanization and selective autophagocytosis of melanosomes. These aberrant processes precede the degeneration of SL melanocytes, but are not sufficient to cause vitiligo without a functioning immune system. They do, however, appear to be involved in provoking an immune response resulting in autoimmune destruction of melanocytes. Loss of melanocytes in the feather is associated with infiltration of lymphocytes and cell-mediated immune activity. Lastly, an environmental component (herpesvirus of turkeys, HVT) also appears to play a role in the development of this disorder in SL chickens (Smyth, 1989; Erf *et al*., 2001).

The SL chickens are unique for studies on the etiopathology of autoimmune diseases, in part, because the target tissue (feathers) is easily accessible and regenerates (allowing for repeated sampling in the same individual), the incidence of the disorder is highly predictable and occurs at a young age and onset and progression of the disease can be visually monitored. Moreover, this avian model for vitiligo offers a unique opportunity to study autoimmune diseases in the context of genetic susceptibility, herpesvirus infection and gender.

Development of the Smyth Line Chicken

The mutant SL (previously known as the delayed amelanosis (DAM) chicken) together with control lines were developed by Dr J. Robert Smyth Jr. at the University of Massachusetts, Amherst, Massachusetts, USA. Their origin has been reviewed extensively, so will only be briefly addressed here (Smyth *et al*., 1981; Smyth, 1989). The progenitor of the SL was one female hatched in 1971 from a non-pedigreed mating of the Massachusetts Brown line (BL). The BL was established in 1963 as a plumage colour-tester line that was homozygous for the e^b allele at the *E* locus. Since this first incidence of amelanosis in 1971, a few cases occurred in each generation of the parent BL, with a current frequency of less than 2%. The basic selection scheme to develop the SL involved efforts concentrated on selection of individuals with

post-hatching pigmentation loss from a central line derived from backcrosses of the original mutant to the BL, as well as, the addition of some amelanotic birds from several outcross F2 populations. When selecting parents for reproduction, individuals with early expression of pigmentation loss as well as varying degrees of severity of vitiligo were chosen. Throughout the years, Dr Smyth developed various BL and SL sublines. Among the criteria used for the development of sublines was the MHC-haplotype, also referred to as the B haplotype (see Chapter 8). Based on MHC typing conducted by Dr and Mrs W.E. Briles at Northern Illinois University, Dekalb, IL, it was found that the BL and the SL carried the same three MHC-haplotypes. Since these haplotypes had not been previously characterized they were designated *B101*, *B102* and *B103*. The *B101* and *B102* haplotypes were also identified in light brown leghorn (LBL) chickens. MHC-sublines (SL101, SL102, SL103, BL101, BL102, BL103 and LBL101) were subsequently developed. The characteristics of these MHC-sublines were summarized by Smyth and McNeil (1999). Unfortunately, most of these valuable lines no longer exist following the closure of the Poultry Farm at the University of Massachusetts and the retirement of Dr Smyth. The lines homozygous for the *B101* MHC-haplotype (SL101, BL101 and LBL101) are the only lines remaining and are currently maintained at the University of Arkansas, Fayetteville, Arkansas, USA by the author. Together, these lines constitute the current animal model for autoimmune vitiligo.

Within this animal model for vitiligo, the SL is considered vitiligo-susceptible, with most individuals expressing vitiligo; the BL is the parental control and is considered vitiligo-susceptible, with a very low incidence of vitiligo; and, the LBL line constitutes the vitiligo-resistant control. The genetic susceptibility/resistance of BL and LBL chickens to vitiligo, respectively, has been further underscored by their response to treatment with the DNA methylation inhibitor 5-azacytidine. Following treatment with 5-azacytidine, 71% of BL chickens developed vitiligo, whereas vitiligo was not observed when the LBL chickens were so treated (Sreekumar *et al*., 1996). Although this treatment caused similar alterations in lymphocyte profiles in primary and secondary lymphoid organs in chickens from the three lines, the only vitiligo-associated effect of 5-azacytidine noted was the infiltration of lymphocytes into the feathers. The phenotypic characteristics of these feather-infiltrating lymphocytes were similar to those observed in vitiliginous feathers from untreated SL chickens (Erf *et al*., 2000). Hence, alterations in gene expression triggered by treatment with 5-azacytidine resulted in recruitment of lymphocytes to the target tissue, loss of self-tolerance to melanocytes and development of an autoimmune response to melanocytes.

The genetic basis of autoimmune vitiligo and line-associated traits has long been described as being under the control of multiple autosomal genes (Smyth *et al*., 1981). A more recent molecular characterization of SL and BL sublines revealed a high level of inbreeding within lines (0.948 for SL101; 0.902 for BL101) and high genetic similarity between SL101 and BL101 lines (similarity index of 0.049 ± 0.006; Sreekumar *et al*., 2001). Hence, it appears that a limited number of genes are responsible for the SL phenotype. With the availability of the chicken genome sequence and other sophisticated bioinformatics and experimental resources for chicken research (i.e. a rich collection of expressed sequence tags from various tissues at different developmental stages, an established dense map of single nucleotide polymorphisms, multiple genomic libraries, pre-made multi-tissue cDNA microarrays and genomic arrays containing 32 773 chicken transcripts and 684 chicken viral transcripts), it is now possible to conduct genome-wide expression analysis and high-resolution quantitative trait loci (QTL) mapping in chickens. These novel tools and approaches together with access to lines such as the SL model that have been kept as closed populations and have alleles predisposing to disease, that are likely fixed or close to fixation, promise effective genetic investigation into the molecular basis of phenotypic traits. QTL mapping using the SL model is currently underway in Sweden by Drs S. Kerje and O. Kämpe (Department of Medical Science) and Dr L. Andersson (Department of Medical Biochemistry and Microbiology) at Uppsala University. These studies will be important in identifying the specific genes responsible for the depigmentation and other abnormalities seen in the SL chicken.

Characteristics of the Smyth Line Chicken

Without experimental manipulation, using standard rearing protocols, 70–95% of the Arkansas SL chickens spontaneously express the vitiligo-like, post-hatch loss of melanin-producing pigment cells (melanocytes) in feather and choroidal tissue. Destruction of melanocytes, which usually is first seen during adolescence and early adulthood in humans, occurs in SL chickens between 6 and 12 weeks of age, but may develop as early as 4 weeks or as late as 18–20 weeks of age (Plate 18.1(a)–(c)). Because regenerating feathers are generally not present in mature chickens, new cases of SLV are rarely observed after 20 weeks of age. In both humans and SL chickens, amelanosis may be either partial or complete, although severe pigment loss is more frequent in the chicken. Re-melanization of amelanotic tissue does occur in both cases, but it is more common in SL chickens. In addition to SLV, SL chickens also exhibit a high incidence of uveitis (<20%, often resulting in blindness), a low incidence of hypothyroidism (<5%, Plate 18.1(g)) and an alopecia areata-like feathering defect (<3%). Similarly, in humans it is not uncommon to find thyroidal, ocular and integumental defects associated with vitiligo (Spritz, 2006). In the SL chicken, the incidence of these associated autoimmune diseases can vary greatly from hatch to hatch and can be increased by selection. Interestingly, mononuclear cell infiltration into the thyroid can be observed without overt clinical symptoms and hypothyroidism in SL chickens can occur with or without SLV (Plate 18.1(g)). On the other hand, blindness and alopecia areata have only been observed in vitiliginous individuals from this line of chicken (Smyth, 1989; Griesse, 2004). Moreover, although not exclusively studied, based on observations by Dr Smyth that the sex-linked dermal shank pigmentation trait (*id+*) may suppress the expression of SLV, we have consistently noted that SL101 chickens with the *id+* phenotype do not express SLV.

Pigmentation and Normal Melanocyte Function in Chickens

In normally pigmented chickens the epidermis of the skin is essentially devoid of melanin and melanocytes. However, melanin is found in other integumental tissues, most notably in feathers and may be present in the beak and scales of the shanks. Dermal melanin is present in the shanks of birds homozygous or hemizygous for sex-linked *id+* and may even be present throughout the dermis in birds that also have the fibromelanotic mutation (Fm; e.g. silky chickens). The melanin-producing cells, destined for integumental or eye tissues, such as the choroid and the anterior surface of the iris, originate in the embryonic neural crest, whereas the iridial and retinal pigment epithelia originate from the outer layer of the optic cup. Undifferentiated melanoblasts from the neural crest migrate early in embryonic development (about 90 h). Those migrating to the feathers populate dermal reservoirs located near the base of the feather follicles, while those migrating to the limb bud remain melanogenically inactive. In growing feathers, portions of the cells in dermal reservoirs migrate into the base of growing feathers via the dermal papillae and the epidermal collar, where they proliferate, differentiate into melanin-producing melanocytes and populate the epithelium. Production of melanin (melanogenesis) by melanocytes is similar in birds and mammals, involves key enzymes like tyrosinase and tyrosinase-related protein-1 and -2 (TRP-1, DHICA oxidase and TRP-2, dopachrome tatomerase, respectively) and occurs in membrane-bounded organelles called melanosomes. Once situated, the cell bodies of melanin-producing melanocytes can be observed, aligned with the interface between the feather pulp and the barb ridge, whereas their dendrites extend along the barb ridge barbule cells (keratinocytes; Plate 18.1(d) and (e)). Pigment (eumelanin and pheomelanin) containing melanosomes are then transferred from the melanocyte dendrites to the barbule cells. As the feather grows and pigment has been deposited in the barbule cells, the melanocyte degenerates and is phagocytosed by keratinocytes (reviewed in Bowers, 1988).

Target Cell Defects

Although SL chicks hatch with a competent pigment system (Smyth, 1989), ultrastructural studies conducted by Boissy *et al.* (1983) revealed that after hatching, regenerating feather melanocytes

produced abnormal melanosomes in SL chickens compared to controls. The abnormal melanosomes had irregularly shaped surfaces containing pigmented extensions that were continuous with the outer rim of the melanosome and appeared to be delimiting an electron-lucent region. Based on histochemical analysis of feather tissue, SL melanocytes were found to have an aberrant and widespread distribution of tyrosinase compared to controls, suggestive of a hyperactive process of melanization in SL melanocytes.

In the feather epithelial barb ridge, one of the earliest manifestations of SLV detected with a light microscope was the appearance of histologically abnormal melanocytes. These melanocytes had thickened, partially retracted dendrites and an irregular shape. Pigment cell transfer from melanocyte dendrites to barbule cells was reduced at this stage. More advanced stages were represented by marked clumping or the absence of melanocytes and further reduction in pigment transfer. Intracellular changes correlating with these abnormal melanocytes included fewer pigmented extensions on melanosomes and aggregation of melanosomes. Eventually, aggregated melanosomes were inside a single large autophagocytic complex, presumably in an effort to contain the cytotoxic melanin precursors. Once autophagocytosis was initiated, melanogenesis effectively stopped as indicated by the disappearance of tyrosinase activity and the concurrent increase of acid phosphatase activity in the abnormal, compartmentalized SL melanocyte (Boissy *et al*., 1986). Amelanotic feather tissue that developed after this state was completely devoid of melanocytes (Boissy *et al*., 1983).

Thus, it appeared that the synthesis of abnormal melanin granules with pigmented extensions was related to a hyperactive process of melanization and that this aberrant process, in turn, stimulated the selective autophagocytosis of melanosomes. Similar degenerative processes in melanocytes could also be observed *in situ* in immunosuppressed SL chickens and *in vitro* in neural crest-derived melanocytes from embryos of SL chickens (Boissy *et al*., 1984, 1986).

One frequently reported difference between vitiligo melanocytes and normal melanocytes in humans is a heightened sensitivity of vitiligo melanocytes to oxidative stress due to an imbalance and/or a deficiency in their antioxidant system (Maresca *et al*., 1997). Considering the extensive generation of reactive oxygen species (ROS) during melanogenesis in both humans and chickens (Bowers *et al*., 1994), a genetic defect in the antioxidant protection system of melanocytes may significantly contribute to the pathological lesion in autoimmune vitiligo. Recent studies conducted in our laboratory using SL, BL and LBL embryo-derived melanocyte cultures revealed heightened ROS generation and lipid peroxidation in SL melanocytes compared to control melanocytes. The occurrence of these malfunctions in culture supports the inherent nature of this melanocyte defect. Moreover, heightened ROS production, oxidative damage and altered redox-status were also observed in the target tissue (feather) during active SLV which may exacerbate the pathological progression of SLV (Erf *et al*., 2005).

The reports described above clearly established the existence of inherent melanocyte defects in SLV. However, when the immune system was inhibited *in vivo* (e.g. by bursectomy or cyclosporin A treatment), the inherent melanocyte defect was not sufficient for the pathological progression of pigment cell degeneration and the appearance of vitiligo.

Immunological Mechanisms

From the initial research into the etiopathology of SLV it became evident that the immune system plays an important role in the loss of melanocytes. Histological studies of the lesion revealed a strong association of mononuclear cell infiltration and the degeneration of melanocytes. When melanocyte loss was complete the mononuclear cells could no longer be observed in the affected feathers (Boissy *et al*., 1983). Additionally, the presence of melanocyte-specific autoantibodies was described in SLV chickens, as were immunofunctional differences in SL chickens compared to controls. These differences included heightened antibody production to T-dependent (sheep red blood cells, SRBC) and T-independent antigens (*Brucella abortus*) (Lamont and Smyth, 1984a), lower graft-versus-host responses and wing-web swelling in response to phytohaemagglutinin (PHA) injection (Lamont and Smyth, 1984b), lower *in vitro* proliferative

responses to stimulation with concanavalin A and altered blood lymphocyte profiles in SL compared to BL controls (Erf *et al*., 1995a). It should be noted, however, that the differences in antibody responses to SRBC observed in the Lamont and Smyth (1984a) study were no longer observed when MHC-sublines B101 and B102 were examined (Sreekumar *et al*., 1995).

The importance of the immune system in melanocyte destruction in SLV was further demonstrated through immunosuppression studies such as neonatal bursectomy (Lamont and Smyth, 1981), inhibition of T cell activity via cyclosporin A (Fite *et al*., 1986; Pardue *et al*., 1987) and suppression of inflammatory immune activity with corticosterone (Boyle *et al*., 1987). It was concluded that in the absence of a functional immune system, melanocyte abnormalities, although present in immunosuppressed individuals, were insufficient for the pathological progression of pigment cell degeneration and the appearance of SLV. Additionally, these studies demonstrated involvement of both humoral and cell-mediated immunity (CMI) in SLV, but attributed a more critical role to CMI.

Humoral Immunity

Maternal melanocyte-specific autoantibodies can be detected in chicks from vitiliginous hens only during the first week of life. After this time, melanocyte-specific autoantibodies appear again in the peripheral circulation 1–2 weeks before the onset of SLV. However, the contribution of these autoantibodies to the onset and progression of SLV has not been defined. The autoantibodies cross-react with mouse and human melanocytes, bind to melanocytes within tissues and recognize antigens expressed in the cytoplasm and on the surface of melanocytes and melanoblasts (Searle *et al*., 1993). Specifically, SL autoantibodies recognize mammalian TRP-1 and, based on molecular studies, the avian homologue of TRP-1 (Austin and Boissy, 1995). To further examine the specificity of these autoantibodies, 2D gel electrophoresis and mass spectrometric analyses are underway.

Cell-Mediated Immunity

At the light microscopy level, one of the earliest manifestations of SLV is the morphological alteration in feather and choroid melanocytes. These cytological changes in melanocytes were associated with large numbers of infiltrating mononuclear leukocytes (IML) into the feather follicle and choroid. When melanocyte destruction is complete, the number of IML in the feather pulp return to levels similar to those in controls (Boissy *et al*., 1983). Based on quantitative and qualitative analyses in our laboratory, using immunoperoxidase staining of frozen feather tissue sections and flow cytometric analysis of immunofluorescently-stained pulp cell suspensions, T cells expressing CD3, CD4, CD8, MHC class II molecules and all three types of T cell receptor (TCR$\gamma\delta$, TCR$\alpha\beta_1$ and TCR$\alpha\beta_2$) could be observed in non-vitiliginous and vitiliginous feathers. In non-vitiliginous feathers, these cells were present in much lower numbers, with proportions among T cells subsets similar to those in blood, and their location restricted to the feather pulp. In feathers from vitiliginous SL chickens, substantial T cell infiltration into the feather pulp has been observed as early as 4–6 weeks prior to visible signs of SLV. In feathers undergoing active melanocyte destruction, T cell numbers were nearly 15 times higher than those observed in feathers from non-vitiliginous controls. The IML can be found throughout the feather: in the pulp, primarily in perivascular areas; at the pulp-barb ridge junction, surrounding the melanocyte cell bodies; and deep in the barb ridge along the melanocyte dendrite and barbule cell alignment (Plate 18.1(f)). The proportions of TCR$\alpha\beta_1$ and TCR$\gamma\delta$ cells were much higher and lower, respectively, among IML in SLV feathers compared to control feathers or blood. At onset of SLV, the ratio between $CD4^+$ and $CD8^+$ lymphocytes (CD4:CD8 ratio) in the feather pulp was near 1.0. With onset of SLV, the CD4:CD8 ratio decreased to levels below 0.4 due to increasing numbers of $CD8^+$ cells. The level of MHC class II antigen expression on pulp macrophages was higher in feathers from chickens with SLV than controls, suggesting the presence of inflammatory mediators like interferon-γ (IFN-γ) in vitiliginous feathers (Erf *et al*., 1995b; Shresta *et al*., 1997).

Using northern blot and western blot analyses as well as quantitative real-time RT-PCR measurement of IFN-γ expression prior to, and throughout, the development of SLV, a strong association of IFN-γ with active vitiligo was confirmed supporting a role of Th1 cell activities and CMI in SLV (Plumlee *et al*., 2006). Moreover, melanocyte death was associated with close physical association between melanocytes and $CD8^+$ lymphocytes and occurred by apoptosis (Wang and Erf, 2004).

Taken together, the observations outlined above bear close similarity to those described in human vitiligo lesions (Ongenae *et al*., 2003) and support the presence of CMI activity in the SLV lesion. Lastly, using the *in vivo* wattle swelling response as an indicator of antigen-specific CMI activity, we were able to demonstrate the presence of melanocyte-specific CMI in chickens with SLV but not in non-vitiliginous SL or control chickens (Wang and Erf, 2003). Curiously, this melanocyte-specific CMI in vitiliginous SL chickens was only directed against feather-derived melanocytes but not embryo-derived melanocytes. Preliminary protein analysis did not reveal differences between feather-derived and embryo-derived melanocytes from control lines of chickens, but revealed a missing 43 kD protein band in feather-derived compared to embryo-derived SL melanocytes (Wang and Erf, 2003). This missing protein band does not explain the specificity of CMI for feather-derived melanocytes in vitiliginous SL chickens, but taken together, these observations do suggest an important role for the local feather environment in the differentiation and antigenicity of melanocytes. Studies, including gene expression and proteomic analyses, are currently underway to further examine the development and nature of the anti-melanocyte response in SL chickens with vitiligo.

Vitiligo-associated alterations in the number and proportions among IML were not reflected in the blood of SLV chickens (Erf and Smyth, 1996). The development of SLV was, however, accompanied by changes in the proportions among blood leukocytes, including substantial increases in inflammatory leukocytes (monocytes, heterophils, basophils and eosinophils) occurring 1–2 weeks before, and at first observation of, SLV (Erf and Smyth, 1996). The role of these inflammatory leukocytes has not been examined further but their increase suggests that onset of SLV is associated with an inflammatory response.

SLV-associated mononuclear leukocyte infiltration was not observed in the skin (Erf *et al*., 1995b) where the undifferentiated melanocyte precursor pool is located. A lack of immune activity directed against undifferentiated melanocytes in SLV is also supported by reappearance of pigmented feathers in vitiliginous SL chickens following a moult. In the skin, lymphocytes were, however, present in dermal lymphoid aggregates and were frequently associated with the epidermis surrounding the feather follicle. Compared to controls, SL dermal lymphoid aggregates were larger and more abundant, contained similar numbers of B and T cells, but differed greatly in the proportions of different T cell subsets (e.g. the CD4:CD8 ratio was 1.7 ± 0.2 and 6.9 ± 0.9 in SL and control dermal lymphoid aggregates, respectively). Moreover, the contents of SL dermal lymphoid aggregates varied with the development of SLV; e.g., the CD4:CD8 ratio was highest at the visible onset of SLV. In light of the lack of a lymph node system in chickens, these dermal lymphoid aggregates may serve as a site for the activation and expansion of lymphocytes that play a role in SLV (Erf *et al*., 1997).

Environmental Factors and Gender Effects

Evidence for the role of an environmental factor in the expression of SLV in susceptible SL chickens is based on the observation that the incidence of SLV in chickens hatched and raised in isolation at the University of Arkansas Poultry Health Laboratory (PHL; biosecurity level 2) was only 10% by 20 weeks of age (instead of 70–95%). This observation was followed by several studies which strongly indicated the presence of HVT as a major "environmental" factor in the expression of SLV (Erf *et al*., 2001). HVT is an alphaherpesvirus which is commonly used as a commercial vaccine to protect chickens from Marek's disease caused by serotype 1 Marek's disease viruses (MDV). Serotype 1 MDV are acute-transforming, cell-associated viruses that cause T cell lymphomas, paralysis and numerous mononuclear cell infiltration-associated

lesions (see Chapter 19). On the other hand, HVT is a non-oncogenic serotype 3 MDV isolated from turkeys. In chickens, HVT causes only minor inflammatory lesions characterized by diffuse, light to moderate infiltration by small lymphocytes (Calnek and Witter, 1991). Like other MDV, in chickens, HVT exhibits strong tropism for feather follicles where it can be detected in its latent stage, ranging from 21 to 105 days of age (Holland *et al*., 1998); other ages have not been examined. A strong link between HVT and the expression of SLV is further supported by our findings that: (1) only vaccination with live HVT, not dead (gluteraldehyde-treated) HVT, can trigger the expression of SLV; (2) that HVT vaccination of SL chicks is accompanied by changes in the profiles of the splenic T cell population not observed in vaccinated BL chicks and (3) there is earlier (by 3 days) and higher immune cell infiltration in skin and feathers in HVT-vaccinated SL compared to HVT-vaccinated BL controls.

Considering the tropism of HVT for the feather, it is likely that in SL chicks the local anti-HVT immune response alters the feather environment in such a way that the already inherently defective, potentially immunologically active melanocytes become visible to the immune system provoking a melanocyte-specific immune response. This scenario is currently our working hypothesis regarding the role of HVT as an environmental factor in SLV. The report by Grimes *et al*. (1996) on the presence of cytomegalovirus DNA in depigmented and uninvolved skin from some patients with vitiligo, and its absence in control subjects, suggests that vitiligo may be triggered by a viral infection in some patients. Considering that cytomegalovirus is also a herpesvirus, the herpesvirus connection in the expression of SLV further underlines the similarities between human vitiligo and SLV.

There is little or no gender preference reported for human vitiligo. However, in SL chickens, vitiligo was first observed in females, but the gender differences disappeared with continued selection for SLV. With the establishment of the B101 breeding population in Arkansas, again gender differences were observed with females having a higher SLV incidence than males, particularly when the birds were reared in isolation at the PHL or on a farm where previously no chickens had been reared (University of Arkansas Veterinary Farm Rabbit House). In the original SL population established in isolation at the PHL, out of 40 chickens, 4 females and none of the males developed SLV in isolation. Before the reason for this low incidence of SLV became evident (i.e. no HVT vaccination after hatching), these 4 females were used extensively for establishing the replacement flock, which may explain why the incidence of SLV in unvaccinated chicks reared in isolation increased over the following generations (up to 25%). However, even several generations later, SLV was not observed in unvaccinated SL males reared in isolation and only occasionally in unvaccinated males reared at the "Rabbit-House". In 2004, the SL populations were moved again, this time to the University of Arkansas Poultry Farm. Since the birds have been maintained on the Poultry Farm, the tendency for the previously observed gender differences disappeared.

Evidence for environmental and immunological interactions with gender in SLV also comes from a study examining the role of IFN-γ in the expression of SLV. Administration of recombinant chicken IFN-γ to unvaccinated SL chickens, reared in isolation, twice per week for the first 6 weeks of life, delivered intraperitoneally or subcutaneously, resulted in a vitiligo incidence of 87.5% in females and 0% in males, whereas, the incidence of vitiligo was 25% and 0% in vehicle-injected unvaccinated female and male SL chickens, respectively. Thus, the cytokine milieu appears to have an important role in SLV and its effect may be gender dependent.

Summary

The SL chicken model (including MHC-matched susceptible and resistant controls) offers unique opportunities to study the interplay between genetic susceptibility, immunological influences, environmental factors and gender; all factors that can lead to the development of anti-melanocyte autoimmune activity. The similarities of the clinical manifestations and pathological progression between human and SLV, together with the unique features of the target tissue (easy, non-invasive and repeatable access to the autoimmune lesion), the predictability of the

disease, and the ability to study the expression of this disorder in the absence/presence of an environmental component, make the SL chicken an excellent model for studies on autoimmune vitiligo and other organ-specific autoimmune diseases.

SPONTANEOUS AUTOIMMUNE (HASHIMOTO'S) THYROIDITIS IN OS CHICKENS

Introduction

Hashimoto's thyroiditis (autoimmune thyroiditis) is the most common thyroid disease in humans. It is characterized by autoimmune destruction of the thyroid gland whereby the thyroid parenchyma is diffusely replaced by a lymphocytic infiltrate and fibrotic reaction and, frequently, the formation of lymphoid germinal follicles. Depending on the extent of lymphoid infiltration, thyroid function may only be slightly affected and the patients remain euthyroid or, with severe infiltration, physiological and clinical manifestations of hypothyroidism develop. Patients with Hashimoto's thyroiditis have serum antibodies reacting with thyroglobulin (Tg), thyroid peroxidase and other thyroid proteins. In addition, many patients have CMI directed against thyroid antigens, demonstrable by several techniques. Onset of the disorder is most common in middle-aged individuals. The incidence is about 10–20 times higher in women than in men, with 2% of the general population affected (reviewed in Chistiakov, 2005).

The OS chicken is one of the best and most thoroughly studied animal models for spontaneously occurring autoimmune thyroiditis that closely resembles human Hashimoto's thyroiditis. The onset of thyroiditis is usually before 6 weeks of age and is accompanied by visible effects on growth and development. These include small size, abdominal and subcutaneous fat accumulation and a plump appearance as well as long, silky feathers that continue to grow and exhibit structural signs of thyroxine deficiency (Plate 18.1(h)). Delayed maturation and low reproductive performance also are characteristics of this line whereby most females do not lay eggs unless supplemented with thyroid hormones. Histological examination of the thyroids of OS chickens reveals extensive infiltration by mononuclear cells and germinal centre formation, commencing in the second week after hatching and resulting in almost complete destruction of the thyroid architecture by 1–2 months of age. Although originally more prevalent in females than in males, with continued selection for the expression of autoimmune thyroiditis, the gender gap in the OS disappears and the incidence of spontaneous autoimmune thryoiditis (SAT) is nearly 100% in both genders. Autoantibodies to chicken thyroid antigens, especially Tg, can be detected in the circulation by 2–3 weeks of age. Moreover, the presence of thyroid-specific CMI in chickens with established thyroiditis has been demonstrated using the delayed wattle swelling response to thyroid extract. Like most autoimmune diseases, the expression of SAT and loss of self-tolerance in OS chickens is a polygenic trait involving many components. Included are inherent target cell defects, manifested in part by abnormal MHC class II antigen expression and iodine uptake of thyroid cells; altered immune function, i.e. heightened levels of immune activity and impaired immunosuppression; and altered immunoendocrine communication via the hypothalamic-pituitary axis (e.g. hyporesponsiveness to glucocorticoids). Additionally, iodine levels in food are an important environmental factor in the development of SAT and the severity of SAT can be manipulated with iodine (Kaplan *et al*., 1991; reviewed in Wick *et al*., 2006).

Taken together, the OS chicken is one of the oldest and best established models of spontaneous, organ-specific autoimmune diseases. Based on clinical, histopathological, immunological, serological and endocrinological aspects, it is well accepted by the biomedical community as one of the best animal models for Hashimoto's thyroiditis.

Since the development of the OS chicken, many excellent, comprehensive reviews have been published by researchers in the USA (Cole, Rose, Sundick, Kaplan and co-workers) and Austria (Wick, Hala and co-workers) that describe the development of the OS model and the research efforts into dissecting the genetic susceptibility and multifactorial nature of this spontaneous organ-specific autoimmune disease (Wick *et al*., 1987, 1989, 2006; Kaplan *et al*., 1991; Rose,

1994; Dietrich *et al*., 1999). These works constitute in depth resources beyond the scope of this chapter, however, an update of current research and a summary of key concepts will be provided below.

Development and Characteristics of the OS Chicken

A detailed analysis of the natural history of the OS chicken was conducted by Dietrich *et al*. (1999). This analysis was possible due to the availability of records over four decades, meticulously maintained by the late Dr Randall Cole, Department of Poultry and Avian Sciences at Cornell University, Ithaca, New York, USA, who first identified the abnormalities in the Cornell C strain (CS) and continued to maintain and improve the OS model until 1995.

In 1955, a few pullets within the CS flock of chickens were observed to have small body size, obesity and long and silky feathers. This disorder was subsequently described as hereditary hypothyroidism with an autoimmune component and characteristics resembling Hashimoto's thyroiditis (van Tienhoven and Cole, 1962). Starting with the few affected pullets, a breeding programme was initiated that led to the development of the OS of chickens. The initial focus of this breeding programme was to increase the incidence of the obese individuals to levels sufficient to study the etiopathology and the mode of inheritance. This was followed by efforts to increase the penetrance and severity of the obese trait. Later, the focus of selection also included improving the reproductive capabilities of the stock.

Along with breeding and selection efforts at Cornell University, a flock of OS chickens has been maintained and propagated as "OS-INN" at the University of Innsbruck, Austria, since the early 1970s. Additionally, a subset of CS and OS Cornell chickens was sent to Austria in 1987 and these populations have been maintained separately (designated OS-C) from the OS-INN chickens.

Considering the role of the immune system in this disorder, sublines based on MHC-haplotypes homozygous for *B5*, *B13* and *B15* (Briles *et al*., 1982) were also developed. When the three MHC-defined sublines were first established, the *B* haplotype appeared to strongly influence the development of SAT with OS$B^{13}B^{13}$ and OS$B^{15}B^{15}$ expressing severe disease compared to OS$B^{5}B^{5}$ chickens which exhibited only mild SAT symptoms (Wick *et al*., 1979). This influence of the MHC on the development of SAT became less pronounced in later generations and the *B* haplotype is not considered a pre-requisite for the development of the disease (Hala, 1988).

A study conducted by Dietrich *et al*. (1997) characterizing age-dependent prediction of onset and severity of SAT constitutes the most recent and complete set of data on the kinetics of SAT development in the MHC-sublines. In this study, thyroids were collected from OS$B^{13}B^{13}$C, OS$B^{5}B^{5}$C, OS$B^{15}B^{15}$INN and OS$B^{5}B^{5}$INN sublines at various intervals from embryonic incubation day (EID) 20 to 5 weeks after hatching. Histological sections were prepared and thyroiditis scored as the degree of thyroiditis (% of thyroid gland area with lymphocytic infiltration in the total cross-section). In all sublines, infiltration was not observed on EID 20. In OS$B^{13}B^{13}$C and OS$B^{15}B^{15}$INN, mononuclear cell infiltration was evident by day 2–4 and in OS$B^{5}B^{5}$C by day 5–7. In both sublines, thyroid infiltration had progressed to aggressive thyroiditis by 4–5 weeks. Within the OS$B^{5}B^{5}$INN line a high (similar to the other lines) and low responder groups (degree of thyroiditis <40%) of chickens were identified, supporting a role for non-MHC genes in the severity of SAT and providing opportunity to study underlying mechanisms responsible for varying degrees of severity of SAT on the same MHC background. Moreover, the descriptive data obtained from this study serve as a valuable guide for the design of studies on etiopathological mechanisms of SAT and preventive approaches.

Early genetic analyses conducted by Cole (1966) suggested that several genes are involved in the control of SAT. Further genetic study led to the postulation of the "two essential sets of genes theory" formulated by Hala (1988) that describes the requirement for two sets of genes to regulate SAT susceptibility. One set of genes (most likely two genes) encodes immune system hyperactivity and the other (approximately three genes; one recessive) encodes susceptibility of

the target organ to autoimmune attack. Using modern genomic approaches, progress continues to be made into the identification of genes responsible for SAT in OS chickens (Vasicek *et al*., 2001; Wick *et al*., 2006).

Immunological Mechanisms

Histological examination of the thyroids in OS chickens reveals infiltration of mononuclear cells and the appearance of organized lymphoid aggregates and follicles. As thyroiditis progresses, healthy thyroid tissue is replaced by these infiltrates often resulting in complete destruction of the thyroid glands. Humoral immunity was thought to play a major role in the development of SAT, based on greatly reduced mononuclear cell infiltration in chicks that were bursectomized (either pre- or post-hatching) and the detection of anti-thyroid autoantibodies. When neonatal thymectomy resulted in more aggressive thyroid infiltration, it first appeared that T cells primarily played a suppressor function in SAT, rather than an active destructive role. However, because neonatal thymectomy does not remove T cells that have already entered the periphery, a role for T cells in thyroid destruction cannot be ruled out. In follow-up studies, neonatal thymectomy was accompanied by administration of high doses of anti-T cell serum which led to almost complete abrogation of thyroid infiltration. Hence, it appears that in the OS chicken the first cells to leave the thymus and take up residence at peripheral sites are effector T cells capable of producing the pathological changes of thyroiditis. On the other hand, the regulatory T cells responsible for suppression of the autoimmune attack appear to leave the thymus at a later time. This time course of events explains the more aggressive presentation of thryoiditis in neonatally thymectomized OS chicks. Moreover, the observed abrogation of thyroid infiltration as a result of complete removal of the T cells compartment, including effector and regulatory T cells, clearly supports a central role for CMI in SAT (reviewed in Rose, 1994; Wick *et al*., 2006).

A phenotypic analysis of thyroid-infiltrating cell populations has been possible with the development of monoclonal antibodies to lymphocyte-specific markers (Kroemer *et al*., 1985). These studies have found that over 60% of thyroid IML are T cells and 10% are activated T cells. Although B cells made up a significant proportion of thyroid IML (about 20–30%), they did not appear to be obligatory for the induction of SAT. Moreover, the overwhelming majority (near 90%) of infiltrating T cells express TCR$\alpha\beta_1$ with a smaller proportion expressing TCR$\alpha\beta_2$ or TCR$\gamma\delta$. These proportions among the various TCR-defined cell subsets differed greatly from those among circulating T cells. Selective depletion of TCR$\alpha\beta_1^+$ and TCR$\alpha\beta_2^+$ T cells using repeated injections into embryonic and 1- to 3-week-old chicks of mouse monoclonal antibodies specific for either TCR$\alpha\beta_1$ or TCR$\alpha\beta_2$, resulted in selective reduction of 41% and 87% of TCR$\alpha\beta_1^+$ and TCR$\alpha\beta_2^+$ cells, respectively (Cihak *et al*., 1995). The reduction in TCR$\alpha\beta_1^+$ cells resulted in a more than 50% decrease in thyroid follicle destruction. Selective reduction of TCR$\alpha\beta_2^+$ cells on the other hand did not affect SAT development. These results indicate preferential usage of the TCR Vβ_1 gene fragment by autoreactive T cells in OS-SAT (Cihak *et al*., 1995).

A similar approach was used to dissect the relative contributions of CD4$^+$ and CD8$^+$ lymphocytes in the destruction of OS thyroid follicles (Cihak *et al*., 1998). Although in untreated chickens IML had substantially higher proportions of CD8$^+$ than CD4$^+$ lymphocytes, anti-CD4 treatment completely prevented the development of SAT in OS chickens. After depletion of CD4$^+$ T cells, neither residual CD4$^+$ cells nor any other mononuclear cells infiltrated the thyroid glands. On the other hand, anti-CD8 treatment reduced the severity of SAT but did not prevent the disease. These findings attest to the critical role of CD4$^+$ cells in the onset and development of SAT while suggesting primary involvement of CD8$^+$ lymphocytes in the pathogenesis and progression of thyroid follicle destruction (Cihak *et al*., 1998).

Considering the absence of thyroid infiltration with selective depletion of CD4$^+$ cells, it is likely that thyroid-infiltrating CD4$^+$ autoreactive T cells provide the necessary inflammatory signals to recruit, activate and retain other cells involved in SAT. To gain insight into the production of inflammatory cytokines and chemokines in OS thyroids, IFN-γ, interleukin (IL)-1β, IL-2, IL-6, IL-8, IL-15 and IL-18 gene expression analysis was conducted using thyroid tissue

collected from OS$B^{13}B^{13}$C and CB controls at various times before SAT development using 20 day embryos and 3- to 5-day-old chicks (Kaiser *et al*., 2002). Although some coordinated expression of IL-1 and IL-8 (a major inflammatory cytokine and chemokine, respectively) was found, the most consistent observation was heightened expression of IL-15 mRNA at all time points, in both the spleen and the thyroid. The biological functions of IL-15 are similar to IL-2 and include stimulation of growth and proliferation of T cells, intestinal epithelial cells, natural killer (NK) cells and activated B cells. Considering the biological functions and the early and persistent expression of IL-15 in the OS thyroid, IL-15 appears to play a role driving the onset of SAT (Kaiser *et al*., 2002). IFN-γ, which is a NK and T helper cell 1 (Th1) cytokine that plays a central role in inflammatory CMI activities, was found to be expressed at heightened levels in OS thyroids when the chicks were 5 days of age. The cellular source of IL-15 and IFN-γ in OS thyroids has not been established (Kaiser *et al*., 2002). Based on the kinetics one would expect little lymphocyte infiltration by 5 days (Dietrich *et al*., 1999). However, considering that OS chickens are known to be immunologically hyperactive, it is likely that even a few NK and/or Th1 cells may produce sufficient amounts of IFN-γ to drive the inflammatory cascade towards the development of SAT. Previous analyses of the kinetics of thyroid infiltration appear to have focused primarily on mononuclear cell aggregates rather than immunohistochemical analysis of dispersed lymphocytes. As shown by Hala *et al*. (1996, 2000), non-specific esterase producing macrophages could be detected even in embryonic thyroid tissues. Hence, it is possible that NK and/or Th1 effector cells were present in OS thyroids in sufficient numbers to account for the rise in IFN-γ prior to visible signs of progressive SAT. The initial presence and activation of these effector cells, however, may simply be due to minor stimuli such as minor injury/malfunction of the susceptible target.

Target Cell/Organ Defects

In OS thyroids there appear to be fundamental abnormalities such as reduced growth of thyroid cells *in vitro* (Truden *et al*., 1983) and alterations in the metabolic function of thyroid cells. The OS thyroid gland can function independently of pituitary stimulation as shown by continued uptake of radioiodide during suppression of thyroid stimulating hormone (TSH; Sundick *et al*., 1979). A similar defect was observed in the CS parental strain. Investigations into this phenomenon excluded faulty TSH regulation or thyroid-stimulating antibodies as the underlying cause for this TSH autonomy and the ability of these lines to utilize the iodide for synthesis of T3, T4 and Tg. Evidence also suggests that iodination of Tg is important in disease induction and that iodine level in the food is an important environmental factor in the development and severity of OS-SAT (Wick *et al*., 1989; Bagchi *et al*., 1995, 1996).

In an effort to gain insight into the role of iodine as an environmental factor in the expression of SAT, Bagchi *et al*. (1995) used OS chickens maintained on iodine deficient diets, thereby avoiding the onset of SAT until challenge with iodine (NaI) and subsequent examination of thyroid injury and infiltration. Twelve hours after NaI administration, thyroid injury but not infiltration was observed. Subcellular changes included swelling of mitochondria and the rough endoplasmic reticulum. The affected cells often had ruptured luminal cell membranes and showed clumping of chromatin in the nucleus. The damage was greater with the higher compared to lower dosages of iodine (250 versus 20 μg). There was no evidence of pyknosis, apoptosis or lipfuscin granules in the thyroid epithelial cells and the follicular structure remained fully maintained. Infiltration of mononuclear, but not polymorphonuclear, leukocytes could be observed 24 h after iodine treatment with maximal levels of infiltration occurring at 72 h and onwards. Phenotypic analyses of thyroid IML showed 40% $CD8^+$ cells, 20% $CD4^+$ cells, 22% B cells and 17% macrophages. This IML population profile is similar to that observed in OS thyroids from chickens that developed SAT without dietary manipulation. This thyroid infiltrate was shown indirectly to be specific to Tg, as only 37.5% of chicks tolerized for Tg at hatching and receiving the iodine treatment exhibited signs of thyroid infiltration, compared to 100% of chicks in the non-tolerized, iodine-treated group. Concurrent treatment with NaI and

ethoxyquin, a strong antioxidant, prevented subcellular changes and mononuclear cell infiltration. This protective effect of the antioxidant on acute iodine injury was not due to its potential influence on iodine accumulation, iodine transport and incorporation into protein. Hence, thyroid injury appears to be a critical event in the induction of OS-SAT by iodine (Bagchi *et al*., 1995).

Other factors that could contribute to the increased susceptibility of the OS thyroid gland to immunological attack include: underlying structural alterations of the thyroid architecture and the presence of exogenous or endogenous leukosis viruses (e.g. *ev* 22) that may cause injury, mimic thyroid autoantigenic components or have immunomodulatory effects (Sundick *et al*., 1979; Ziemiecki *et al*., 1988; Kuehr *et al*., 1994). The observation, that thyroid epithelial cells from OS chickens are known to have a lower threshold for IFN-γ-induced expression of MHC class II antigen provides evidence for another functional defect which could greatly contribute to the autoimmune recognition and destruction of the thyroid follicles (Wick *et al*., 1987).

Summary

In the OS chicken, minor inborn errors in metabolism and minor aberrancies in the structure, growth and function of the thyroid tissue may necessitate an influx of macrophages and dendritic cells to regulate tissue homeostasis. This non-infectious influx could, however, be a first step on the road to thyroid-specific autoimmunity. Due to the inherent hyperactive immune response activity known to exist in the OS, an inappropriate high inflammatory cytokine cascade, including heightened IFN-γ production, may stimulate the IFN-γ-sensitive thyroid epithelial cell to become immunologically active (e.g. express MHC II antigen, produce cytokines and chemokines and present self-antigen). This together with other known defects in the OS, such as (1) early emigration of T effector cells from the thymus into the periphery, (2) an imbalance between effector and regulatory T cells and (3) a disturbed endocrine-immune system balance, including hyporesponsiveness to glucocorticoids, may all culminate into autoimmune recognition of thyroid antigens and destruction of the thyroid epithelium (Wick *et al*., 2006). Considering that a similarly complex scenario can be put forward for Hashimoto's thyroiditis in humans (Chistiakov, 2005), OS chickens will continue to provide a unique opportunity to examine the contributing factors and mechanisms in susceptibility and etiology of SAT and for the design of intervention strategies.

SCLERODERMA IN THE UCD-200 AND 206 LINES OF CHICKENS

Introduction

Scleroderma, also known as systemic sclerosis (SSc), is a complex autoimmune connective tissue disease characterized by pathological remodeling of connective tissues. Clinical and pathological features in humans include microvascular alterations, perivascular inflammatory infiltrates and alterations involving both T and B cell compartments, presence of multiple autoantibodies and ultimately widespread tissue fibrosis of the skin and several internal organs. The extent and severity of clinical manifestations range widely; however, a progressive thickening and fibrosis of the skin is universally observed in patients with SSc. Internal organ involvement tends to be subclinical at presentation but may involve fibrosis of the oesophagus, lungs, heart and pericardium, kidneys, thyroid and the male reproductive system. In advanced stages, progression of the vascular and fibrotic changes is accompanied by a decrease in inflammation. Scleroderma is more prominent in females than males and is believed to result from complex interactions between the host's genetic background and the environment. Several other factors have been proposed as agents triggering/modulating the expression of scleroderma; included are exposure to organic solvents and toxins both at home and in the work place, the presence of microchimerism, infectious agents such as human cytomegalovirus and the inherent tendency for oxidative stress with associated generation of oxidative radicals (Abraham and Varga, 2005).

The UCD-200/206 lines of chicken spontaneously develop an inherited disease closely resembling human SSc (Plate 18.1(i) and (j)). It is considered the best animal model as it occurs naturally and exhibits the whole spectrum of clinical, histopathological and serological manifestations of human SSc (i.e., vascular occlusion, severe lymphocytic infiltration of the skin and viscera, fibrosis of the skin and internal organs, antinuclear antibodies, rheumatoid factors and distal polyarthritis). Moreover, together with MHC-matched and non-related control lines of chickens, this animal model provides excellent opportunity to examine genetic susceptibility and etiopathological mechanisms of SSc, as well as preventative and intervention therapies (Wick *et al*., 2006).

Development and Characteristics of the UCD-200 and 206 Lines

Dr Paul Bernier at the Department of Poultry Husbandry, Oregon State University, Corvallis, Oregon, first reported that male chickens showed signs of dermal fibrotic disease reminiscent of scleroderma in 1942. In 1977, the UCD-200 line was developed at the UCD and first described by Gershwin *et al*. (1981). Thereafter, the UCD-206 line was developed which is homozygous *B15* and MHC-matched with the UCD-058 and H.B.15FIN normal white leghorn lines that serve as healthy controls. As described by Wick *et al*. (2006), a UCD-200 colony was established in the Experimental Animal Facilities of the Innsbruck Medical University in 1988, followed by a colony of UCD-206 chickens in 1993. With the loss of these valuable lines of chickens at the UCD, the colonies at the Innsbruck Medical University are the only source of UCD-200/206 currently available.

UCD-200/206 chickens follow a relatively uniform pattern of disease progression (Van de Water *et al*., 1995; Wick *et al*., 2006). The chicks appear relatively normal during the first 1–2 weeks after hatching. The first observable gross abnormalities include severe swelling and erythema, leading to necrosis and loss of the comb. This typical "self-dubbing" comb lesion occurs in more than 90% of UCD-200/206 chicks (Plate 18.1(i)). By 3–4 weeks of age, dermal lesions in the dorsal neck region, such as swelling, induration and loss of feathers become evident in 20–40% of the chicks (Plate 18.1(j)). With progression of the disorder, the skin becomes thickened and tight. Histological examination has shown early skin inflammation that is later replaced by fibrosis of the dermis and subcutaneous fat and muscle. Alterations in internal organs such as oesophagus, small intestine, kidney, lungs and testis can also be observed. The age and incidence of internal organ involvement may vary widely with alterations in renal arterioles occurring in almost all of the chickens.

Originally, the incidence of SSc in UCD-200 chickens was higher in males than females, but with continued selection this gender preference has disappeared. Based on genetic analyses, the genetic defect responsible for SSc in UCD-200 chickens appears to be autosomal and recessive and exhibits incomplete penetrance (Gershwin *et al*., 1981). Additionally, a modulatory role for the MHC-haplotype was indicated by backcrossing experiments of UCD-200 ($B^{17}B^{17}$) to other MHC-haplotypes (Abplanalp *et al*., 1990) which resulted in lower disease penetrance with some MHC-haplotypes, but not with *B15* (UCD-206 chickens are $B^{15}B^{15}$). Genomic analyses of collagen and endogenous virus loci using both the UCD-200 and 206 lines revealed no gross alterations of collagen genes, which is consistent with observations in humans. Although a novel endogenous leukosis virus (*ev* 23) was detected in the UCD-200/206 lines, its role in the development of avian SSc is not clear (Sgonc *et al*., 1995).

Immunological Mechanisms

In UCD-200 chickens, dermal pathology of both the comb and neck integumental tissue involves prominent mononuclear cell infiltration. Initially, T cells including both $CD4^+$ and $CD8^+$ lymphocytes, predominated in the lesions, but as the lesions progressed distinct groups of IgM^+ B cells could be observed. Further phenotypic analysis of skin infiltrates in the early acute phase revealed that the overwhelming majority of skin IML in the deeper dermis and subcutaneous tissue consisted of $TCR\alpha\beta_1{}^+$, $CD3^+$, $CD4^+$, MHC class II^+ T cells with only 5–10% expressing the IL-2 receptor (IL-2R). T cells in the inflammatory infiltrate present in the perivascular region

of the papillary dermis consisted primarily of TCR$\gamma\delta$ MHC class II$^+$ lymphocytes (Van de Water *et al*., 1995; Gruschwitz *et al*., 1993). These observations strongly support T cell-mediated immune activity in the SSc lesion.

Compared to healthy control chickens, alterations in the proportions and numbers of T cells in the blood and thymus, as well as altered T cell functional activities (decreased *in vitro* T cell mitogen-induced proliferation, IL-2 production and IL-2R expression, abnormal function of lymphocyte co-stimulator molecules or intracellular calcium regulators) also point towards T cell abnormalities and a role of T cells in UCD-200 SSc (Gruschwitz *et al*., 1993). Immunohistological study of thymic tissue from UCD-200 chickens revealed alterations suggesting impaired T cell maturation already prior to visible signs of disease onset. These included profound defects in thymic structure with depletion of type 1 epithelium lining the subcapsular and perivascular regions, excessive MHC class II expression particularly in the cortex, and increased IL-2R expression by thymocytes (Boyd *et al*., 1991; Wilson *et al*., 1992). These selective abnormalities in the thymic environment associated with avian SSc, together with more recent observations of reduced numbers of apoptotic thymocytes in UCD-200 thymii compared to controls (Sgonc and Wick, 1999) suggest altered T cell maturation and selection potentially influencing establishment, maintenance and regulation of tolerance.

Although in human and avian SSc a major role of T cells in the pathogenesis is widely accepted, autoantibodies are also present in both (Abraham and Varga, 2005). Circulating autoantibodies in UCD-200/206 chickens consist of: antinuclear antibodies (ANA) including single-stranded DNA, poly (I) and poly (G) and anticardiolipin antibodies, anti-cytoplasmic antibodies and rheumatoid factors, as well as, anti-endothelial cell antibodies (AECA). The levels of these autoantibodies increase with age and, with the exception of the AECA, their role in the development of SSc is not clear (Haynes and Gershwin, 1984; Gruschwitz *et al*., 1993). AECA in UCD-200/2006 chickens are found before the onset of SSc and have been shown to be involved in endothelial cell injury (Sgonc *et al*., 1996; Worda *et al*., 2003).

In both human and avian SSc, endothelial cells have emerged as the primary target of the autoimmune response. As a result of autoimmune attack, endothelial cells undergo apoptosis (Sgonc *et al*., 1996). In human SSc, AECA induce endothelial cell apoptosis as a result of NK cell-mediated antibody-dependent cell cytotoxicity and Fas–Fas ligand interaction (Sgonc *et al*., 2000). Similarly, a role of UCD-200 AECA in endothelial cell apoptosis has been demonstrated (Worda *et al*., 2003). However, in both human and chickens, the specific autoantigens recognized by AECA autoantibodies have not yet been identified (Wick *et al*., 2006).

Endothelial cell apoptosis is followed by accumulation of mononuclear cells and fibrosis (Nguyen *et al*., 2000). Compared to controls, fibroblast lines prepared from fibrotic skin of UCD-200 and 206 chickens demonstrated an activated phenotype (heightened production of collagen, non-collagenous protein and glycosaminoglycan) and, similar to neoplastic fibroblasts, increased expression of highly branched *N*-linked oligosaccharides terminating in *N*-acetylglucosamine residues (Chechik and Fernandes, 1992; Duncan *et al*., 1992).

Mononuclear cells isolated from fibrotic skin of UCD-200/206 chickens secrete pro- and anti-fibrotic cytokines and IgM and, hence, likely play an important role as effector cells in the development of dermal fibrosis (Duncan *et al*., 1995). Although the identity of the cytokines was not known, a recent study by Prelog *et al*. (2005) suggests that diminished transforming growth factor (TGF)-β2 production leads to increased expression of a profibrotic procollagen alpha 2 type I mRNA variant in embryonic fibroblasts of UCD-200 chickens, providing another glimpse into the complexity of the interrelationships underlying this systemic autoimmune disease.

Summary

Considering the many similarities between human and avian SSc, UCD-200 and 206 lines of chickens will continue to make significant contributions to our understanding of SSc in humans. SSc in UCD-200/206 develops spontaneously, predictably and early, with both dermal and

other organ involvement. This provides excellent opportunities to dissect the complex interrelationship between genetic susceptibility, immune system defects and environmental factors that drive the onset and progression of this disease. Additionally, information obtained by studying healthy chickens in comparison to those affected by this multi-organ disorder will greatly contribute to our understanding of poultry biology.

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19 TUMOURS OF THE AVIAN IMMUNE SYSTEM

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INTRODUCTION
TUMOURS OF THE IMMUNE SYSTEM
ONCOGENIC MECHANISMS OF TUMOUR VIRUSES
IMMUNE RESPONSES TO ONCOGENIC VIRUSES
ANTI-TUMOUR RESPONSES
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INTRODUCTION

The immune system of birds, as in the case of mammals, consists of a complex network of a large number of cell types and cascades of soluble factors, all of which work in concert to induce protective immune responses against the continuous challenge from a wide range of pathogens. A properly functioning immune system is vital to the survival of commercial poultry flocks kept in intensive conditions in the poultry house environment, where they are constantly challenged by a plethora of rapidly spreading infectious pathogens. An optimum immune system is also critical to induce effective immune responses to the wide range of live and inactivated vaccines that protect against a very large number of poultry pathogens.

As detailed in other chapters, the avian immune system has made invaluable contributions to the understanding of the many fundamental principles of immunology (see Chapter 1). Among these, perhaps the most significant contribution is the demonstration of the dichotomy of the adaptive immune system into the T- and the B-cell-dependent compartments that are associated with the cell-mediated and humoral immune responses, respectively. T- and B-lymphocytes, the essential component cells of the vertebrate immune system, develop through the primary lymphoid organs thymus and bursa of Fabricius, respectively, before populating in the secondary lymphoid organs such as the spleen, bone marrow and local lymphoid tissues. These cells face up to the challenge of the pathogenic organisms and, especially in a vaccinated host, respond vigorously either as cytotoxic T-lymphocytes (CTL) or T-helper (Th) cells or as antibody-secreting B-cells to eliminate invading pathogens and to provide continuing protection through memory cells. Apart from lymphocytes, other cell types such as macrophages, dendritic cells (DC) and natural killer (NK) cells also function as major players in combating infection either as part of the innate immune responses or antigen-presenting cells. Thus, the avian immune system plays a crucial role in preventing disease and maintaining health. However, as is the case with other body systems, the immune system is also vulnerable to different types of diseases, and many

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pathogens specifically target this system with devastating effects. In this chapter, some of neoplastic diseases of the avian immune system are described.

TUMOURS OF THE IMMUNE SYSTEM

Neoplastic diseases in poultry can be of infectious and non-infectious aetiology. The non-infectious tumours, although important in terms of animal welfare, are not of major economic significance since they are usually sporadic, mostly occurring in birds older than the usual lifespan of production poultry. On the other hand, infectious neoplastic diseases caused by oncogenic viruses are very widespread and hugely important economically. The three neoplastic diseases mainly affecting the avian immune system are (1) Marek's disease (MD)-associated T-cell lymphomas, (2) avian leukosis tumours of B-cells and other haematopoietic cells, and (3) reticuloendotheliosis virus (REV)-induced tumours characterized by a variety of syndromes including lymphoid neoplasia (Payne and Venugopal, 2000). In addition, lymphoproliferative disease (LPD) in turkeys has also been reported in some parts of the world as an infectious neoplasm involving the lymphoid organs. Comprehensive reviews covering the various aspects of the diseases caused by these pathogens are available elsewhere (Fadly and Payne, 2003; Witter and Fadly, 2003; Witter and Schat, 2003; Davison and Nair, 2004).

Marek's Disease

MD, named after the Hungarian pathologist József Marek, is a lymphoid neoplasia of domestic chickens and, less commonly, turkeys, quails and geese. Originally described in 1907 as a polyneuritis affecting the peripheral nerves, it was not until 1926 that the disease was recognized as a neoplastic disease associated with tumours in several visceral organs. Although MD exists in all poultry-producing countries of the world, assessment of the worldwide incidence and economic impact is difficult as it is not a notifiable disease. Nonetheless, a recent survey has indicated that MD still remains a major problem in many countries (Gimeno, 2004). The most recent estimates of economic impact of MD on the world poultry industry is in the range of US$1–2 billion annually (Morrow and Fehler, 2004). MD is not vertically transmitted through infected eggs. However, chicks become infected with the virus almost immediately after hatching from the heavily contaminated poultry houses. The infection of chicks occurs by inhalation of the infected desquamated epithelium in poultry house dust, shed from the feather follicle epithelium of infected birds. The poultry house dust can remain infectious for long periods because of the high stability of the virus. Cytolytic infections occur between 3 and 6 days after infection leading to extensive atrophic changes in the bursa of Fabricius and thymus causing some early mortality. After this early cytolytic period, the virus becomes latent, and the clinical expression of paralysis or tumours can occur anytime after 3–4 weeks after infection. Under field conditions, most of the serious cases begin after 8–9 weeks, but sometimes commence well after the onset of the egg production. Various factors such as virus strain and dosage, gender and genetic resistance of the host, presence of maternal antibodies and environmental factors all can affect the outcome of infection.

Clinical signs associated with MD vary according to the specific syndromes and can be divided broadly into different clinical forms based on the various characteristics. In almost all these cases, these involve lymphoid infiltrations into tissue(s) to produce lesions or tumours. In the classical form of the disease, mainly neural lesions occur with mortality rarely exceeding 10–15% over a few weeks or many months. Signs can vary from bird to bird depending on the involvement of the different nerves. The most common clinical sign is the partial or complete paralysis of the legs and wings. When nerves controlling the neck muscles are affected, symptoms such as torticollis are observed. Similarly, the involvement of the vagus nerve can result in the paralysis and dilatation of the crop. Such birds could also show symptoms of gasping and respiratory distress. In the acute form of the disease, where there is usually formation of

lymphomas in the visceral organs, the incidence of the disease is frequently between 10% and 30% and in major outbreaks can increase to 70%. Apart from generalized manifestations such as depression, weight loss, anorexia and diarrhoea, the clinical signs are less marked. Mortality can increase rapidly over a few weeks, and then cease, or can continue at a steady rate or decline over several months. Acute cytolytic disease observed with some of the recent very virulent MD virus (vvMDV) strains, shows a severe atrophy of the lymphoid organs. This form of the disease, sometimes also described as "early mortality syndrome", results in very high mortality usually between 10 and 14 days of age. Transient paralysis is a rather uncommon manifestation of MDV infection that occurs between 5 and 18 weeks of age. Affected birds suddenly develop varying degrees of ataxia, paresis or paralysis of the legs, wings and neck. The disease is commonly observed 8–12 days after infection and usually lasts only for about 24–48 h, and is associated with oedema of the brain. The affected organs show lymphoid infiltrations, with the degree of infiltration correlating with the disease manifestations.

The unique epidemiological features of MD such as the highly contagious nature, the widespread distribution and the long-term infectivity of the poultry house environment, make the eradication of the disease almost impossible. Hence, the control of the disease is essentially based on preventive vaccination (see Chapter 20), although improved biosecurity and genetic resistance can contribute to the control. The development of MD vaccines was a significant landmark both in avian medicine and basic cancer research, as this was the first example of a neoplastic disease controlled by the widespread use of a vaccine (Calnek, 1992). Vaccination with live attenuated vaccines represents the main strategy for the prevention and control of MD. These are usually administered as cell-associated vaccines to day-old chicks to provide protection against the natural challenge the chicks are exposed after leaving the clean hatchery environment and being placed in the infected poultry house environment. With the introduction of *in ovo* immunization methods, an increasing number of birds are vaccinated by this route. MD vaccines, derived from all the three MDV serotypes, are highly effective often achieving over 90% protection under commercial conditions. The most widely used serotype 1 vaccine is derived from the CVI988/Rispens strain and is effective against most of the vvMDV and vv+MDV pathotypes. Antigenically related serotype 2 strains such as SB-1 and 301B/1 are also used widely in many countries. The serotype 3 FC-126 strain of herpesvirus of turkey (HVT) is available as cell-free and cell-associated forms of vaccines. Although many of these vaccines are effective individually, the concept of protective synergism (Witter and Lee, 1984; Lee *et al.*, 2003) has led to widespread use of polyvalent vaccines with two or more strains administered simultaneously. Although MD vaccines are generally successful in controlling losses, vaccine failures can occur from causes such as (i) improper use of the vaccine, (ii) exposure to virulent viruses before the development of immunity, (iii) interference by maternally derived antibodies and (iv) the emergence of virulent viruses that can breakthrough the immunity (Nair, 2005).

Avian Leukosis

Avian leukosis embraces several different leukaemia-like neoplastic diseases of the haematopoietic system. These tumours are induced by avian leukosis virus (ALV), members of the *Alpharetrovirus* genus of the family *Retroviridae* (Regenmortel *et al.*, 2000). Structurally, ALV possesses, from the 5′ end to the 3′ end of their genome, three structural genes, *gag/pro-pol-env*, which encode, respectively, the proteins of the virion group-specific (gs)-antigens and protease, the enzyme reverse transcriptase enzyme and the envelope glycoproteins. On the basis of the viral envelope, ALV are grouped into 10 subgroups designated A–J, of which chickens are the natural hosts for the subgroups A, B, C, D, E and J (Venugopal, 1999). On the basis of the mode of transmission, they can be further grouped into "exogenous" and "endogenous" retroviruses. ALV that are transmitted from bird to bird, either through the egg or contact are termed "exogenous" viruses and all oncogenic ALV fall in this category. However, some ALV are present as integrated proviruses in the genome of normal birds and are transmitted genetically as Mendelian genes, either as complete viral genomes (e.g. infectious virus of subgroup E) or, more commonly, as incomplete

(defective) genomes coding for retroviral products (e.g. gs-antigen) only. Such viruses are termed "endogenous" ALV. These are generally non-oncogenic although they may influence the response of the bird to infection by exogenous ALV by inducing immunological tolerance or immunity.

Depending on the primary cell type transformed, avian leukosis can be grouped into different types of tumours: lymphoid leukosis (LL), tumours of B-lymphocytes, is one of the commonest forms of leukosis. LL occurs in chickens from about 4 months of age and is most commonly caused by ALV of subgroups A and B. Gross pathological changes include diffuse or nodular enlargement of the bursa of Fabricius, liver, spleen and other organs due to coalescing foci of extravascular immature lymphoid cells. Erythroid leukosis or erythroblastosis is an uncommon, usually sporadic, tumour of the erythroid cells occurring mainly in adult chickens. The disease is an intravascular erythroblastic leukaemia. In the affected birds, the liver and spleen, and sometimes the kidneys, are moderately and diffusely enlarged and often of bright cherry-red colour. Microscopically the liver shows intra-sinusoidal accumulations of rather uniform, round, erythroblasts, the spleen shows accumulations of erythroblasts in the red pulp, with bone marrow showing enlarged sinusoids filled with erythroblasts. Myeloid leukosis broadly involves both myeloblastic myeloid leukosis (myeloblastosis) and myelocytic myeloid leukosis (myelocytomatosis). The disease has become particularly prevalent in broiler breeders infected with subgroup J ALV in many countries. ALV are also associated with a variety of solid tumours, including fibrosarcoma, chondroma, haemangioma, histiocytic sarcoma, mesothelioma, myxoma, nephroblastoma, osteoma and osteopetrosis.

ALV induce tumours by two main types of mechanisms: (1) by the activation of a cellular proto-oncogene after ALV integration. This leads to the activation of cellular oncogenes leading to neoplastic transformation, the mechanism being described as "insertional mutagenesis". Examples of such oncogene activation include the c-*myc* in LL (Hayward *et al.*, 1981) and c-*erbB* in erythroblastosis (Maihle *et al.*, 1988). As this mechanism of induction usually takes several weeks or months these viruses are termed "slowly transforming" viruses. (2) By the activation of a transduced oncogene carried by the ALV genome. Such viruses are able to induce tumours rapidly and are termed "acutely transforming" viruses. Examples of such viruses include MC29 that carry v-*myc* (Vennstrom *et al.*, 1982) and myeloblastosis viruses that carry v-*myb* (Perbal *et al.*, 1986) oncogenes.

ALV can be transmitted either by vertical (congenital or egg) or by horizontal spread through contact. In the vertical transmission, the eggs become contaminated with the virus within the oviduct leading to the infection of chick embryos during incubation. This route of congenital infection leads to strong associations between the presence of virus in vaginal swabs, egg albumen and embryos, which provide the basis for ALV eradication programmes in breeding stock. Congenitally infected chicks are an important source of contact infection in the hatchery and during the brooding period, and meconium and faeces from congenitally infected chicks contain high concentrations of ALV. The horizontal mode of spread is responsible for the high incidence of infection in flocks. Sources of virus from infected birds include faeces, saliva and desquamated skin. Unlike MDV, the survival of ALV outside the body is relatively short and hence eradication of the virus is feasible in many farms.

Eradication of ALV from a flock depends on breaking the vertical transmission of virus from dam to progeny and preventing re-infection of the progeny. The procedures for eradication depend on the identification and elimination of hens that shed ALV to their egg albumen and hence to their embryos and chicks. Such hens, which are viraemic and shedders of the virus, are usually identified by testing their cloacal/vaginal swabs or egg albumen by enzyme-linked immunosorbent assay (ELISA) for presence of high levels of ALV gs-antigen. Continuous monitoring and elimination of infected birds will break the spreading life cycle of the virus and lead to ALV eradication (Payne, 1998).

Reticuloendotheliosis

Reticuloendotheliosis refers to a group of syndromes in poultry and game birds associated with REV (Witter and Fadly, 2003). REV, belonging to the family *Retroviridae*, shows no serological

relationship to ALV. One of the syndromes associated with REV is the runting disease syndrome characterized by runting, bursal and thymic atrophy, enlarged peripheral nerves, abnormal feather development, proventriculitis, enteritis, anaemia, and liver and spleen necrosis. The abnormal feathering, in which the barbule of wing feathers are adhered to the feather shaft, is termed "nakanuke" in Japanese, and has been seen in chicken flocks vaccinated with REV-contaminated vaccines. REV also induces chronic lymphoid neoplasm in the bursa of Fabricius and other organs. These tumours are of B-cell origin and are caused by REV proviral insertional activation of the cellular *myc* oncogene. Non-bursal lymphomas of T-cell origin have also been induced experimentally with latent periods as short as 6 weeks involving the thymus, liver, heart and spleen. Acute reticulum cell neoplasia (reticuloendotheliosis) is induced by the defective T-strain of REV that carries the v-*rel* oncogene (Bhat and Temin, 1990). When injected into young chicks, the T-strain, originally isolated from a turkey with leukotic lesions, induces rapid proliferation of primitive mesenchymal or reticuloendothelial cells causing death within 1–3 weeks (Hoelzer *et al.*, 1980). REV is transmitted both horizontally by contact with infected chickens and turkeys, and vertically from tolerantly infected chicken and turkey dams. Vertical transmission can also occur from infected male chickens and turkeys. REV can be detected in faeces and cloacal swabs. REV gs-antigen, and sometimes infectious virus, can be detected in albumen of eggs from tolerantly infected hens.

Because of the usually sporadic and subclinical nature of REV infections in chickens and turkeys, large-scale control procedures have generally not been considered necessary in commercial poultry production. However, freedom of infection in breeding flocks that produce progeny for export is required by some importing countries. It is considered probable that REV eradication could be achieved by prevention of vertical transmission by detecting shedding dams and sires for REV gs-antigen by ELISA, and rearing progeny in isolation, using methods similar to those for control of leukosis.

ONCOGENIC MECHANISMS OF TUMOUR VIRUSES

In eukaryotes, the normal processes of cell division, differentiation and death are controlled in a tightly regulated and coordinated fashion, thanks to the highly complex integrated, intricate molecular circuitry of growth regulatory pathways. Such tight regulation keeps vital checks on cell proliferation and destroys any damaged or mutated cells by physiological processes such as apoptosis. Neoplastic transformation is the end result of the disruption of one or more of these critical pathways which leads to the uncontrolled cellular proliferation and inappropriate survival of damaged cells. Understanding the specific molecular pathways associated with oncogenesis is often difficult because of the complexity and the integrated nature of these regulatory networks. As efficient inducers of neoplastic transformation, oncogenic viruses exploit the weaknesses of the regulatory networks to surpass the rigid checks on cell proliferation resulting in uncontrolled cell proliferation and neoplasia. In this respect, oncogenic viruses have been some of the greatest allies of the oncologists to gain necessary intelligence on the molecular mechanisms of induction of neoplasia. In the past few decades, studies on the molecular events and interactions in cells infected by different oncogenic viruses have helped us to understand some of the critical pathways of oncogenesis. This includes the remarkable demonstration of activation and/or "capturing" of cellular genes by retroviruses to induce cellular transformation, which also led to the discovery of the first oncogene v-*src* (Vogt and Hu, 1977). Several DNA viruses, through the molecular interactions of their viral proteins, also have contributed to the fundamental insights into oncogenic mechanisms (O'Shea, 2005). These include the mechanisms of transformation by viruses such as the simian virus SV40 through its large T-antigen, adenoviruses through their E1A/E1B proteins, human papilloma viruses (HPV) through their E5/E6/E7 proteins and Epstein–Barr virus (EBV) through the various nuclear antigens. Many of these viral gene products target critical points to disrupt the molecular events in the regulation of cell cycle, transcription, tumour suppressor genes and apoptosis (O'Shea and Fried, 2005).

Oncogenic Mechanisms of Retroviruses

Contributions of avian retroviruses to the fundamental understanding of the mechanisms of neoplastic transformation have been remarkable. The initial description of transmission of tumours by Ellerman and Bang (1908), followed by the Nobel Prize winning discovery of direct transmission of tumours through filtrates of tumour extracts by Peyton Rous (1911), paved the way for elucidating some of the intricate molecular mechanisms of oncogenesis. Other Nobel Prize winning discoveries by Harold Varmus (1990) and Michael Bishop (1990) on the activation of proto-oncogenes by avian retroviral integration led to our understanding on the role of oncogenes in neoplastic transformation.

Oncogenic retroviruses can be broadly grouped into (a) slowly transforming or non-acute viruses and (b) acutely transforming viruses that differ markedly in oncogenicity, tumour tropism and oncogenic mechanisms.

Non-acute Retroviruses

These groups of viruses are widespread in avian species and cause a variety of cancers affecting lymphocytes and other haematopoietic cell types. All these viruses are replication competent and contain a full complement of viral genes flanked by two long terminal repeats (LTR) at each terminus that function as strong promoters for the expression of viral RNA. These viruses induce tumours by integrating the proviral DNA at or near the host proto-oncogenes or genes involved with growth control. This process referred to as insertional mutagenesis has been extensively reviewed (Kung and Liu, 1997). As a result of the proviral integration and the LTR promoter function, the proto-oncogenes are transcriptionally deregulated, contributing to the genetic alterations that unleash growth control leading to neoplastic transformation. The tumours that develop by this process are usually monoclonal or oligoclonal and occur after long incubation periods. Examples of this method of retroviral oncogenesis include the induction of LL by ALV subgroup A/B (Hayward *et al.*, 1981; Neiman *et al.*, 2006) and ML by ALV subgroup J (Chesters *et al.*, 2001) through insertional activation of c-*myc*.

Acute Retroviruses

Compared to the slow onset and mono/oligoclonal nature of the tumours induced by non-acute retroviruses, acute retroviruses can cause rapid-onset polyclonal tumours. Many of the acute retroviruses are able to transform cells *in vitro* to produce transformed foci or colonies in cell cultures, providing a convenient assay for their identification. This ability of the acute retroviruses to transform cells *in vitro* and produce tumours *in vivo* is due to the presence of viral oncogenes in their genomes. These oncogenes are transduced by the viruses from the host genomes by a process of recombination (Kung and Liu, 1997). During subsequent selection of tumour phenotypes, many of these oncogenes acquire mutations that enhance their oncogenic potential. In nearly all cases, transduction of oncogenes results in deletion of parts of the viral genome mak ing these viruses replication defective. The structure of the defective viral genome differs between viruses. However, all these viruses require helper viruses for their propagation. The potential of acute retroviruses as very potent carcinogens to induce rapid-onset polyclonal tumours is related to the high level of expression of oncoproteins mediated by the viral LTR and continuous selection of mutations that enhance the oncogenic function. As these properties are carried by the viral genome, there is no requirement for specific viral integration. A large number of acute retroviruses carrying a wide range of viral oncogenes have been identified. At the functional level, the majority of the viral oncogenes transduced by retroviruses are associated with the signal transduction pathways. Examples of acute retroviruses associated with transformation of avian haematopoietic cells include MC29 virus containing v-*myc* (Vennstrom *et al.*, 1982), MH2 virus containing v-*myc* and *mil* (Coll *et al.*, 1983; Saule *et al.*, 1983), and avian myeloblastosis virus carrying the v-*myb* oncogenes (Perbal *et al.*, 1986). More recently, acute-transforming ALV-J strain 966 that induces rapid-onset myeloid leukosis was also shown to express v-*myc* as a gag-*myc* fusion protein (Chesters *et al.*, 2001).

Oncogenic Mechanisms of DNA Tumour Viruses

DNA viruses belonging to papovavirus, adenovirus, hepadnavirus and herpesvirus families have been associated with tumours in man and animals (O'Shea, 2005). Compared to the RNA tumour viruses which activate host genes or use transduced host genes to transform their cell targets, most oncogenic DNA viruses encode their own proteins to induce neoplastic transformation. Many of these viral proteins, despite their diverse origin, act by interfering with the cell functions at critical points of the cellular regulatory network. Some of the examples of such interactions include those of the SV40 large T-antigen, adenovirus E1A/E1B and HPV E5/E6/E7 proteins interfering with tumour suppressors p53 and retinoblastoma (O'Shea and Fried, 2005).

Among the oncogenic DNA viruses that induce tumours of the cells of the avian immune system, MDV is the most important. This highly contagious alphaherpesvirus belonging to the genus *Mardivirus* induces rapid-onset T-cell lymphomas in poultry (Osterrieder *et al.*, 2006). The induction of T-cell tumours in genetically susceptible birds occurs after a robust early cytolytic infection and a period of latent infection in lymphocytes. Although latency is generally considered a prerequisite for oncogenic transformation of the target T-cells, only a proportion of latently infected cells is thought to be transformed and will develop into tumours. The molecular mechanisms that drive latently infected cells to transformation and subsequently into aggressive neoplasia are not fully understood (Nair and Kung, 2004).

One of the recent advances in understanding the oncogenic mechanisms of MDV is the determination of the complete genome sequence of several viral strains. MDV genome contains more than 100 potential open reading frames (ORF), which include both those that are unique to MDV as well as those which are homologous in other herpesviruses (Osterrieder and Vautherot, 2004). Genome-wide sequence comparisons between virulent and attenuated strains together with the analysis of viral gene expression in MDV-transformed tumour-derived cell lines have indicated that the genes within the RL region (repeats flanking the unique long regions) are most likely to be associated with oncogenicity of the virus. These include the major oncogenesis-associated genes such as the *M*DV *Eco*RI-*Q* (MEQ) fragment and the virus-encoded RNA telomerase (vTR) subunit. Detailed review on the molecular mechanisms of the functions and roles of these genes in MDV oncogenesis are available elsewhere (Nair and Kung, 2004).

Wild-type MEQ is a 339-amino acid transcriptional factor that plays a major role in MD pathogenicity. Through its leucine zipper motif, MEQ can either form homodimers with itself or form heterodimers with proteins such as c-*Jun*, *JunB* and c-*Fos*. It has been proposed that the upregulation of the various transformation-associated genes by MEQ heterodimers, together with upregulation of anti-apoptotic factors such as Bcl-2 and v-*ski*, is fundamental to the oncogenicity of MDV (Kung *et al.*, 2001; Levy *et al.*, 2005). In addition to the dimerization with other leucine zipper proteins, MEQ can also actively interfere with the functions of several cell cycle control genes including p53, retinoblastoma and cyclin-dependent kinase 2 (CDK2), which would contribute to the oncogenic transformation of the T-cells. Similarly, MEQ-induced upregulation of CD30, a member of the tumour necrosis factor receptor II (TNFR II) family, demonstrates an interesting parallel to those seen in some of the human lymphomas including Hodgkin's lymphoma (Burgess *et al.*, 2004). Further evidence of convergent evolution of the molecular mechanisms of herpesvirus-induced oncogenesis is provided by the critical requirement of interaction between MEQ and the transcriptional co-repressor carboxyl-terminal-binding protein (CtBP) in MDV-induced oncogenesis (Brown *et al.*, 2006). Similar interactions of CtBP with the EBV proteins EBNA3A and 3C are also crucial for efficient transformation of human B-cell by EBV (Hickabottom *et al.*, 2002).

In addition to MDV-encoded proteins, non-coding regions in the MDV genome are also being recognized as important elements in oncogenesis. One such unique element that was recently shown to have profound effect on neoplastic transformation of T-cells and oncogenesis is the vTR identified in the IR_L/TR_L region of the MDV genome (Fragnet *et al.*, 2003, 2005). vTR has extensive secondary structures similar to the EBV-encoded small RNAs (EBERs) produced during latent EBV infection. Chicken telomerase RNA (chTR) subunit, which shows

88% sequence identity to vTR, forms a functional core complex of the ribonucleoprotein telomerase, an enzyme that has been implicated in cellular transformation and tumorigenesis. Recent studies using deletion mutants have clearly demonstrated that vTR has an important role in MDV-induced malignant T-cell tumorigenesis (Trapp *et al.*, 2006). More recently, distinct classes of small microRNAs, encoded by MDV have been identified (Burnside *et al.*, 2006). These virus-encoded microRNAs, transcribed from the region flanking the MEQ gene as well as the LAT region of MDV, are expressed at very high levels in MD tumours suggesting that they play major roles in oncogenesis.

IMMUNE RESPONSES TO ONCOGENIC VIRUSES

The immune system has primarily evolved to eliminate extraneous agents. In order to achieve this, the host has developed a number of immunological mechanisms, which can be broadly grouped into innate and adaptive responses (see Chapter 7). The innate responses are the first line of defence against infections and are mediated through different pathways such as the interferon system, the complement and the NK cells that recognize and destroy infected cells. The adaptive responses that appear later are specifically directed against pathogens and consist of humoral and cell-mediated responses. Immunological memory also features in adaptive immune responses, when pools of cells that are reactive with a particular pathogen are maintained as primed memory cells. This enables the immune system to encounter the same or closely related organism with a more rapid and robust immunological response by activating this memory cell pool. Immune responses to avian oncogenic viruses are distinctly unique in that these responses are directed against the very cells that are involved in the immune responses.

Immune Responses to Leukosis Sarcoma Viruses

Humoral responses mediated through specific neutralizing antibodies are the essential components of immunity against retroviruses. This can clearly be demonstrated in congenital egg-transmitted ALV infections, where absence of neutralizing antibodies is usually associated with high levels of viraemia and virus shedding (Fadly and Payne, 2003). The majority of these antibodies are directed against the epitopes on the surface of the viral envelope. Such antibodies act by inducing conformational changes in the viral envelope blocking receptor binding or early post-binding events. Antibody responses have been demonstrated against both gp85 and gp37 subunits of the retroviral envelope. However, subgroup-specific neutralization of ALV is associated with the gp85 subunit of the envelope, particularly with the five clusters of variable regions within this domain. Antigenic variants that escape virus neutralization show mutations within this region (Venugopal *et al.*, 1998; Venugopal, 1999). Some of the non-neutralizing antibodies can also affect retrovirus infections through antibody-dependent cell cytotoxicity (ADCC) (Lamon *et al.*, 1977).

Major histocompatibility complex (MHC)-restricted CTL-mediated immune responses have also been identified against retroviral infections. The best example of this is the difference in susceptibility to Rous sarcoma virus (RSV)-induced tumours by different haplotypes of chicken lines (Taylor *et al.*, 1994; Taylor, 2004). While some genetic lines of chickens inoculated with the RSV develop tumours that progress inducing death, RSV-induced tumours show regression in other lines (Pinard-van der Laan *et al.*, 2004; Praharaj *et al.*, 2004). The regression of tumours in the resistant lines is determined by a dominant gene, *R-RS-1*, that lies within the MHC locus (Taylor, 2004). Conserved peptide motifs of the RSV proteins that bind to the MHC have been shown to be protective against RSV tumour growth in chickens with the B-F12 haplotype (Hofmann *et al.*, 2003). More recently, the peptide motifs of the single dominantly expressed class I molecule associated with the MHC-determined responses to RSV have been identified (Wallny *et al.*, 2006). Some influence of the lymphocyte antigen Bu-1 locus on RSV tumour regression and of the Th-1 locus on LL is reported (Bacon *et al.*, 1985; Fredericksen

and Gilmour, 1985). The importance of cell-mediated immunity in the regression of RSV tumours has also been documented in other studies. For example, $CD8^+$ T-cells isolated from birds recovered from v-*src*-induced sarcomas were able to protect naïve birds (Gelman *et al.*, 1993). Similarly, neonatal thymectomy prevented regression of tumours in chickens and quails (Yamanouchi *et al.*, 1971; Yoshikawa *et al.*, 1976).

Other cell types such as NK cells and macrophages also may play a role in the immune responses against RSV tumours. For example, it has been demonstrated that ALV-transformed cell lines such as LSCC-RP9 could be lysed by NK cells (Sharma and Okazaki, 1981). Similarly, macrophages from RSV tumour regressor lines of chicken showed more cytotoxicity on these cell lines demonstrating the role of these cell types in immunity against RSV tumours (Schnegg *et al.*, 1994). The importance of the interaction between the MHC and CTL in ALV has been demonstrated using RCAS vector expressing chicken MHC class I (Thacker *et al.*, 1995). In addition to its use in the assessment of the CTL response to ALV, this system has also been extremely useful tool in evaluating CTL responses to other viral disease in chickens.

Immune Responses to REV

As in the case of ALV, neutralizing antibodies play an important role in immunity to REV. The antibodies against gp90 of the virus are thought to be the most important in protection, as recombinant fowlpox virus (FPV) expressing the glycoprotein protected against viraemia after challenge (Calvert *et al.*, 1993). In addition to the humoral immune responses, cell-mediated immunity is considered to be a major element in REV infection, as REV has been used as an important model for studying virus-specific, MHC-restricted CTL responses in chicken (Omar and Schat, 1997; Markowski-Grimsrud and Schat, 2002, 2003). $CD8^+$ CTL against REV have been identified, although the importance of the *in vivo* responses in REV infection has not been ascertained (Merkle *et al.*, 1992). However, neonatal thymectomy increased REV-induced mortality in birds challenged with the T-strain virus, suggesting that cell-mediated responses are important for protection (Linna *et al.*, 1974). Cell-mediated immune responses and NK cells may also influence anti-REV immune responses (Weinstock *et al.*, 1989).

Immune Responses to MDV

As with most other pathogens, infections with MDV also result in the activation of innate and acquired immune responses (Schat, 1991). However, MDV also has major immunosuppressive effects on the host, as described in Chapter 16. The importance of the equilibrium between immune responses and immunosuppression cannot be overemphasized; a distortion in the balance towards immunosuppression often will lead to the disease. The immune responses developing during the early cytolytic phase are crucial for the outcome of infection, since any impairment of immune responses during this phase could delay the establishment of latency prolonging the cytolytic destruction of immune cells by virus-induced apoptosis. The immune response during the latency phase is also important for preventing onset of MD lymphomas. The vaccine-induced immunity is thought to be primarily an anti-tumour response, since MDV vaccines do not prevent superinfection with the virus. However, vaccines do reduce cytolytic infection thereby preventing extensive damage to the immune system through the continued destruction of immune cells. Innate immune responses against MDV include changes in cytokine expression as well as NK and macrophage responses. These include the upregulation of a number of pro-inflammatory cytokines driving a Th-1-type response, the increased transcription of inducible nitric oxide synthase II (iNOS), as well as the enhanced NK cell and macrophage activity (Schat and Xing, 2000).

Specific acquired immune responses, both humoral and cell mediated, have been described after natural infection or vaccination with MDV. The importance of humoral immune responses in the immunity against MD is considered to be relatively minor due to the highly cell-associated character of the virus. However, the presence of maternal antibodies may delay virus replication

and interfere with vaccine-induced immunity especially when cell-free vaccines are used. Virus-neutralizing (VN) antibodies can be induced by MD vaccination/infection, and these are mostly thought to be directed against the antigens such as the glycoprotein B.

Cell-mediated responses mediated through specific CTL have been identified as critical components of the immunity against MD as early as the late 1970s (Ross, 1977). The crucial role of $CD8^+$ T-cells in controlling MDV infection has been confirmed using CD8-deficient chickens (Morimura *et al.*, 1998). The role of MHC-restricted, antigen-specific CTL in immune responses against MD has also been demonstrated using REV-transformed MHC-defined lymphoblastoid chicken cell lines stably expressing individual MDV genes such as phosphoprotein (pp)38, glycoprotein (g)B, gC, gH, gE, gI, MEQ, infected cell protein (ICP)4 and ICP27 (Schat and Xing, 2000).

ANTI-TUMOUR RESPONSES

In addition to the major role in protecting against the multitude of pathogens that constantly threaten our health, a properly functioning immune system is also vital in the fight against neoplastic diseases. Neoplastic transformation is the result of a dysregulated proliferation resulting from a multistep process involving mutational events in multiple genes such as tumour suppressor genes, transcriptional regulatory genes or proto-oncogenes. As neoplastic transformation is an ongoing process, recognition and elimination of tumour cells by the host defences is vital for maintaining the homeostasis. One of the recognized outcomes of the anti-tumour immune responses is the "spontaneous" tumour regression observed in many species including birds. Anti-tumour immunity stems from the ability of the immune system to recognize and destroy individual cells of the tumour. The arms of the innate, as well as the acquired, immune system are thought to participate in anti-tumour immunity.

In order for a tumour cell to elicit an immune response, the B- and T-lymphocytes must recognize specific epitopes from the antigens expressed on the tumour cells. These tumour antigens, either intra- or extra-cellular, are presented in an immunological context with MHC class I by the antigen-presenting cells such as the DC to naïve T-cells, which under appropriate cytokine environment are activated into various forms of effector cells (see Chapters 9 and 10).

The tumour antigens recognized by the immune system in this manner can be either derived from the host itself or can be viral antigens in the case of virus-induced tumours. The host-derived tumour antigens include: (a) differentiation antigens (e.g. carcinoembryonic antigen) which are upregulated at an incorrect time in ontogeny; (b) tumour-specific shared antigens (e.g. melanoma-specific MAGE) which are normally expressed only in immunologically privileged sites; (c) tumour-specific mutated antigens (e.g. mutated *ras*) and (d) overexpressed normal antigens (e.g. HER-2/neu). Examples of virus-derived tumour antigens include EBV LMP-2 or HPV E7 antigens (Young and Rickinson, 2004).

In the past, identification of tumour antigens has been achieved by various techniques such as the expression cloning of tumour cell-derived cDNA libraries. Serological expression cloning of tumour antigens (SEREX) identifies tumour-derived expressed cDNA libraries on the basis of reactivity to syngeneic CTL clones or autologous serum (Nakatsura *et al.*, 2002). Novel tumour antigens have also been identified from the sequences of specific peptides eluted from MHC class I expressed on tumour cells. More recently, DNA microarray and proteomics techniques that compare the gene expression between tumour cells and healthy cells of the same lineage are being used for identification of novel tumour-associated antigens (Chatterjee and Zetter, 2005).

Compared to the immune responses against avian tumour viruses, very little data are available on the nature and significance of anti-tumour responses. Regression of RSV tumours in genetically resistant birds is strongly associated with anti-tumour immune responses directed against *src* (Hofmann *et al.*, 2003; Wallny *et al.*, 2006). In MD, it is often suggested that vaccine-induced immunity is predominantly an anti-tumour response, although vaccines also

have a clear effect on reducing early cytolytic infection. Regression of MD tumours as evidence of anti-tumour immunity has been demonstrated (Sharma *et al.*, 1973; Burgess *et al.*, 2001), although the mechanisms largely remain unclear. A group of antigens, generally referred to as Marek's disease tumour-associated surface antigens (MATSA), have been identified on MDV-transformed cells (Matsuda *et al.*, 1976; McColl *et al.*, 1987; Pradhan *et al.*, 1991). The majority of these antigens are considered to be markers of T-cell activation. One such MATSA, recognized by the monoclonal antibody AV37 (Burgess and Davison, 2002), has recently been shown to be the chicken homologue of CD30, a member of the TNFR II family (Burgess *et al.*, 2004). Increased expression of the CD30 on MDV-transformed tumour cells (Ross *et al.*, 1997) and detection of specific anti-CD30 immune responses in infected MD-resistant chickens suggests that immunity to CD30 could be a mechanism of anti-tumour responses against MD lymphomas.

In summary, the tumours of the immune system caused by the avian oncogenic viruses constitute a major source of economic loss to the poultry industry. In addition to providing a better understanding on the pathogenesis of these diseases, studies on these tumours have given the opportunities to gain fundamental insights into various facets of the avian immune system. Since a well-functioning immune system is crucial both for responding to the challenges from multitude of pathogens and for generating adequate immune response to the cocktail of vaccines administered in the lifetime of a chicken, effective control measures against tumours of the immune system are vital for preventing direct losses from these diseases.

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20 PRACTICAL ASPECTS OF POULTRY VACCINATION

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INTRODUCTION
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INTRODUCTION

The immune system evolved to free the multicellular host from noxious pathogens (Janeway, 1992). Similar to the mammalian immune system, the immune system of the chicken, as the best-studied representative of birds, is a multilayered network of cells and molecules, which are active at different points in time with different roles and interactions. Similar to most mammals, the chicken immune system can be divided into an innate and an adaptive defence system. Both are necessary for vaccination-induced immunity as outlined below.

The immune system of vertebrates consists of multiple interconnected cell types with different capacities and functions, which interact by cell-to-cell contacts and by secretion of a variety of autocrine or paracrine molecules. While some cell types are sessile, others are motile and circulate through the blood and the lymphatic system in order to detect antigen presented in lymph nodes, which collectively drain any tissue in the body. As such, this defence system can be regarded as an elaborate system, which is functionally divided into an early responding, innate and a slow-reacting adaptive immune system. The two systems are essential and need to cooperate for antimicrobial, primary and vaccination-induced immunity, as discussed in Chapter 7. Innate immune cells include epithelial cell, macrophages, dendritic cells (DC), various granulocytes and natural killer (NK) cells. They are able to respond within minutes. The adaptive system, comprising T and B lymphocytes, requires several days to weeks for activation and needs clonal

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TABLE 20.1 Key Characteristics of Innate and Adaptive Immune Responses

	Innate	Adaptive
Specificity	Oligospecific cells, expressing evolutionary conserved, germline encoded receptor	Monospecific cells, expressing highly variable clonally distributed receptors
Speed	Immediate response	Delayed response
Memory	No	Yes

expansion in order to produce the desired cellular responses and effector molecules. Because the innate immune cell carries multiple receptors it is oligospecific. For example, a macrophage carries receptors for microbial structures such as lipopolysaccharide (LPS), various lipopeptides, virus-associated double- or single-stranded RNA, or specific DNA motifs. This limited set of conserved molecular patterns is characteristic for the microbial world and conserved for entire classes of pathogens. On the other hand, T and B lymphocytes carry just one receptor specific for its cognate antigen (see Table 20.1 for a general overview). Bridging between innate and adaptive immunity is mediated by innate antigen-presenting cells (APC), activated upon recognition of conserved structures of microbes. These cells also recognize, process and present antigen fragments to adaptive T cells, which are essential for efficient downstream priming and regulation of adaptive immunity (see Chapter 9).

Apart from cell-to-cell interactions, locally active, secreted cytokines comprise the master regulators of the immune system (see Chapter 10). They are expressed in waves and act in an autocrine or paracrine fashion. Genetic deletion studies in knock-out mice have demonstrated considerable redundancy for numerous cytokines when addressing immune function phenotypes. On the other hand, certain steps within the immune cascade show absolute dependence of particular cytokine activities, including lymphoid organization and T helper (Th) cell polarization.

IMMUNOLOGY OF VACCINATION

Different pathogens require distinct types of host immune reactions. When designing a vaccine, it is preferred to know beforehand the desired type of adaptive immune reaction(s), i.e. the so-called immunological correlate of protection (IMCOP). The adaptive immune system can be subdivided in two major arms: humoral (antibody-based) and cellular or cell-mediated reactions. For certain micro-organisms antibody-dependent immunity is sufficient, while for others cell-mediated immunity is essential. Also a combination of both may be preferred – sometimes at different times after infection. Functional evidence for a certain IMCOP is confirmed by either adoptive transfer of immune cells or antiserum. For example, purified T cells or T cell subpopulations on the one hand or B cells or immune antibodies (as B cell products), on the other, are transferred into a naïve recipient, which is subsequently exposed to microbial challenge and monitored for level of transferred resistance. Alternatively, the IMCOP can be determined by selectively depleting certain immune elements suspected of providing protection against subsequent microbial challenge. A decrease in immunity in the depleted group confirms an essential role in protection, while normal resistance, when compared to the non-depleted control group, suggests a lack of contribution to immunity. In poultry both humoral and cell-mediated immune (CMI) reactions are essentially similar to mammalian counterparts.

As mentioned earlier, vaccination aims to protect the host from disease upon exposure to noxious micro-organisms. This is achieved successfully if the host has generated readily available immune effector elements such as antibodies that are able to immediately recognize and neutralize the relevant pathogen. If antibodies are unable to neutralize the microbe, for example because it has an intracellular life cycle, CMI reactions should be programmed to eliminate the

infected target cell before spreading of the microbe can take place. This occurs by increasing the frequency of antigen-specific memory T cells, which rapidly expand upon secondary recognition of antigen(s) of the invading micro-organism. Immunological memory forms the basis for protective vaccines (see the section on stimulation of immune memory in this chapter).

Ideally, the desired type of adaptive IMCOP required for a particular pathogen should be known because the type of immune response induced by a vaccine largely depends on the composition and type of vaccine. For example, the vaccine can be a whole, inactivated micro-organism or its purified or recombinant subunit formulated together with an immunopotentiator. Alternatively, the antigen may be expressed by a harmless viral or bacterial vector or an expression plasmid, the so-called DNA vaccine. Classically, it may also be a live-attenuated strain of the pathogen.

Upon inoculation of the vaccine preparation initially innate immune cells will be triggered. For efficient priming of adaptive immune reactions it is important to first stimulate APC, especially DC. DC are excellent APC for naïve T cells and therefore critical for primary immune induction. They are regarded as the sentinels of the immune system, which take up, process and present antigen to T cells, thereby bridging innate and adaptive immune reactions. In addition, they become activated by distinct signals (see below) and codeliver so-called costimulatory molecules for T cells.

IMMUNE RESPONSE POLARIZATION

As mentioned before, there is evidence in poultry for the existence of both humoral immunity and CMI. Involvement of antibodies can be easily measured, while a role for CMI is often concluded by default. In mammals, the selection of effector immune responses is controlled by antigen-specific Th cells, which may secrete Th type 1 or type 2 characteristic cytokines at different proportions (see Chapter 10). Th1 cytokines, such as interferon (IFN)-γ, promote CMI resulting in cytotoxic T lymphocytes (CTL), natural killer (NK) cells and macrophage activation for cytolysis or phagocytosis, while Th2 cytokines interleukin (IL)-4, IL-5 and IL-13 support B cell responses and antibody production. However, IFN-γ may also contribute to antibody production especially of the immunoglobulin (Ig)G2a isotype subclass in mice (Huang *et al*., 1993). Mutually exclusive Th cell polarization, with associated cytokine diversity, was first observed in mice (Mosmann *et al*., 1986), resulting from cross-inhibitory transcriptional regulation of cytokine synthesis and receptor downregulation (Abbas *et al*., 1996). Later a similar skewed response was shown in humans although with a less pronounced polarization (Romagnani, 1991). After cloning and characterization of the first homologues of signature cytokines in porcine, bovine and companion animals it became apparent that the cytokine-driven skewed immunity also holds for most mammalian species of veterinary relevance (Schijns and Horzinek, 1997).

By contrast, in chickens, and presumably avian species in general, only the orthologues of Th1 type cytokine, including IFN-γ and IL-18 could be identified, mostly with a limited (30–35%) amino acid identity to mammalian orthologues (Staeheli *et al*., 2001). The functional homologue of chicken (ch)IL-12, driving IFN-γ synthesis and splenocyte proliferation, was identified only very recently (Degen *et al*., 2004). However, until recently no evidence was found for Th2 type cytokines in chicken cDNA libraries with over 300 000 expressed sequence tags (EST). Based on the high synteny between the chicken and human gene clusters, Kaiser and co-workers succeeded in identifying the first non-mammalian Th2 cytokine gene sequences, and provided evidence for a non-functional, pseudo IL-5 gene sequence (Avery *et al*., 2004). However, chickens lack IgE and IgG isotype class switching and possess fewer eosinophils, basophils and mast cells, all typical hallmarks of mammalian Th2 responses. Evidence for cytokine-polarized immunity in birds remains scanty. Recently, Degen *et al*. (2005b) provided evidence for an avian equivalent of polarized cytokine profiles, i.e. Th1-dominated responses, characterized by elevated production of the signature cytokines IFN-γ and IL-12, typically triggered by Newcastle disease virus (NDV) infection in chickens, while selectively augmented IL-4 and IL-13 gene expression was noted during helminth (*Ascaridia galli*) infection, associated with diminished IFN-γ expression.

TABLE 20.2 Immunopotentiator Classification Based on Function

Signal	Key Process	Mechanism	Effect
Signal 1 facilitators	Antigen geography (location)	Transport of antigen towards the secondary lympoid organs	Immunoavailability
	Pulsed or prolonged antigen release (time)	Prolonged/repeated delivery of antigen	
	Antigen structure (form)	Particulate, macromolecular structure of antigen	
Signal 2 facilitators	Stranger (signal 0)	Recognition of microbial non-self	Immune activation
	Danger	Activation after tissue damage	
	Inhibition of natural immune attenuators	Blocking natural inhibition	

Similar to mammals, in chickens induction of adaptive immune responses is controlled by help from antigen-specific $CD4^+$ Th cells (Arstila *et al*., 1994). However, existence of avian Th1 and Th2 type subpopulations needs to be demonstrated. Mammalian Th1 and Th2 type responses are controlled by $CD4^+CD25^+$ T regulatory (Treg) cells, which can suppress the activities of both Th1 and Th2 populations. The counter-balancing activity of Treg cells, characteristically producing IL-10 and transforming growth factor (TGF)-β, results in more even-handed immune responses, avoiding uncontrolled proliferation and exacerbated (auto) immune reactivity. In general, *in vivo* adaptive immune responses like IgG isotype switching by B cells and stable CTL development depend on concomitant interaction with and activation of antigen-specific Th cells in secondary lymphoid organs (Mondino *et al*., 1996).

Appropriate priming of Th cells is preceded by proper activation of APC, belonging to the innate immune system. They enter lymphoid organs through afferent lymph after sampling of antigen in the peripheral tissues. DC are especially crucial APC for primary Th cell priming and act as a bridge between the innate and adaptive immune responses, as described in Chapter 9. Activation of APC may occur through specialized receptors recognizing non-self, microbial "stranger" signals or endogenous "danger" molecules from damaged tissues, according to current thinking (Janeway, 1992; Matzinger, 1994). The stranger and/or danger signals induce upregulation of costimulatory and major histocompatibility complex (MHC) molecules, a switch in chemokine expression and migration to the draining lymph nodes. Absence of costimulatory molecule expression (signal 2) results in T cell anergy and death and T cell tolerance to the antigen. Hence, APC activation is a key step in activation of adaptive immune reactions. On the other hand, prolonged or repeated residence of stable antigen may also lead to immune activation according to another hypothesis (Zinkernagel *et al*., 1997). For a more detailed explanation please see below and also the section on immune activation and immunopotentiation, as well as Table 20.2. For the chicken it is likely that, proper activation of innate immune reactions is essential for subsequent triggering of adaptive immunity. Although likely, there is lack of direct evidence for similar priming of Th cells via DC in chickens.

Antibody Production

Antibodies contribute to microbe neutralization by complement activation and/or opsonization by NK cells, macrophages and monocytes (see Chapter 7). In mammals, opsonization occurs following Fc receptor binding to the constant domain of the Ig heavy chain of IgG, IgE and IgA isotypes (Clynes *et al*., 2000). In poultry, similar processes are assumed to take place. However, chickens exhibit fewer isotypes, limited to homologues for IgM, IgG (named IgY) and IgA. Details on the

Ig of other domesticated species are presented in Chapter 21. In mammals, naïve IgM^+, IgD^+ B cells pass secondary lymphoid tissues such as spleen and lymph nodes in B cell-rich follicles. After recognition of cognate antigen these cells migrate towards T cell-rich areas to interact with antigen-specific $CD4^+$ T cells and receive T help, before they can proliferate and differentiate into plasma cells. Especially, production of IgY antibodies normally requires help from antigen-specific $CD4^+$ Th cells (Arstila *et al*., 1994). Activation of Th cells requires presentation of the same antigen by matured APC. The upstream events leading to APC activation and maturation will be discussed later. Antibodies are considered essential protective elements induced by many existing vaccines (Parker, 1993). Antibodies are induced by the vast majority of avian vaccines though these do not necessarily correlate with protective immunity. The subtype induced, the level of response and duration of response are influenced by the type of vaccine used.

Cell-Mediated Immune Responses

In both mammals and chickens, elimination of many intracellular pathogens is assumed to critically depend on $CD8^+$ CTL which recognize antigen-derived peptides presented with self (MHC) molecules on the surface of infected cells. Classical CTL assays are tedious and time-consuming and require MHC-matched target cells. The relative ease of measuring antibodies and the relative lack of tools to adequately measure CMI reactions has hampered investigations into the involvement of CMI in protective immunity in poultry vaccinology. As a result, relatively little is known about the relevance of CTL responses for the control of infectious poultry diseases (Schat, 1994), although CTL are considered essential for elimination of Marek's disease virus (MDV) (Schat and Markowski-Grimsrud, 2001), avian influenza virus (AIV) (Seo *et al*., 2002) and salmonella (Beal *et al*., 2006). Recently, we investigated the contribution of antibody formation upon vaccination with an attenuated chicken reovirus strain in protective immunity to challenge infection (van Loon *et al*., 2003). We found no decrease in immunity in vaccinated B cell depleted animals, suggesting that cellular immunity is sufficient for protection of broilers under such conditions.

In mammals, CMI is often associated with a certain cytokine profile. Most Th1-associated cytokines in chickens are now identified, and can be monitored as immunological correlates, either by cytokine-specific enzyme-linked immunoabsorbent assays (ELISA) in supernatants of *ex vivo* antigen-restimulated cells, or by enzyme-linked immunospot (ELISPOT) assays, and possibly even by MHC class I or II tetramer staining (Wallny *et al*., 2006). On the other hand, classical cell lysis assays can be established to measure functional CTL activity (Maccubbin and Schierman, 1986; Schat, 1994; Seo and Collisson, 1997). In general, however, these cell culture-based assays are more difficult to establish and to reproduce. More general information is obtained by quantitation of *in vivo* cytokine gene expression, at cellular or organ specific level, by quantitative or semi-quantitative RT-PCR (Degen *et al*., 2005a) or by microarray analysis (Degen *et al*., 2006).

Responses to Live Vaccines

The immune response induced by a live vaccine is influenced by the type of micro-organism, the degree of attenuation, the delivery route, the site of replication as well as the age and immune status of the bird. Live vaccines in regular use within the poultry industry include those with anti-viral, -bacterial and -parasitic specificity. Due to their ability to replicate in the host, live vaccines induce a variety of innate and adaptive immune responses, the onset, magnitude, duration and quality of these responses being associated with the unique characteristics of the particular vaccines. Generally speaking, peptides derived from intracellular replication of micro-organisms are presented on the cell surface by MHC class I molecules to $CD8^+$ T cells, giving rise to CTL responses, when presented by APC. Peptides from micro-organisms that replicate in intracellular vesicles, or from ingested bacteria are presented by class II MHC to $CD4^+$ T cells (see Chapter 9). These cells can be differentiated into Th1 or Th2 cells, both of which can initiate the humoral

immune response by activating naïve B cells to produce IgM. However, as outlined above, there is little evidence for Th2 type responses in chickens.

In general, the live vaccines in use by the poultry industry have been attenuated by serial passage in tissue culture, eggs or embryo-derived tissues, with the aim of maintaining the immune response induced by the parent organism whilst attenuating the ability of the micro-organism to cause disease or immunosuppression.

The Innate Immune Response to Live Vaccines

The use of live vaccines in the poultry industry reflects the requirement for an early onset of immunity with the chicks sometimes exposed to field challenge shortly after vaccination. With increasing knowledge of the avian innate immune response (see Chapter 7) it is becoming clear that many of the live viral, bacterial and parasitic vaccines induce rapidly acting innate immune responses. For example, vaccination against Marek's disease can protect the chick within a few days, this protection is believed to initially be associated with NK cell activity (Sharma, 1981; Heller and Schat, 1987), expression of IFN-α (Sharma, 1989) and IFN-γ (Xing and Schat, 2000) and possibly activation of macrophages (Schat and Xing, 2000). The innate immune response is also likely to play an important role in the early onset of immunity associated with live vaccines against viral respiratory diseases (Fulton *et al*., 1993; Jeurissen *et al*., 2000; Lambrecht *et al*., 2004), infectious bursal disease virus (IBDV) (Jeurissen *et al*., 1998), salmonella (Wick, 2004; Kogut *et al*., 2005) and coccidiosis (Lillehoj, 1998). Analysis of the chicken genome plus the use of genomics and proteomics will help the further characterization of the innate responses to live vaccines (Avery *et al*., 2004; Karaca *et al*., 2004; Roach *et al*., 2005).

Humoral Responses to Live Vaccines

Humoral responses to live vaccines have been well characterized. The type and duration of antibody response is dependent on the micro-organisms used and the route of vaccine delivery. Systemic vaccination with MDV vaccines provides a broad-spectrum serum antibody response (van Zaane *et al*., 1982) though the relevance of this response in vaccine-induced immunity is unclear. In contrast, vaccinations against viral respiratory diseases via a mucosal route, for instance infectious bronchitis virus (IBV) and NDV live vaccines, induce local IgA in the Harderian gland (Davelaar *et al*., 1982; Russell and Koch, 1993), lacrimal IgA and IgM (Davelaar *et al*., 1982; Russell, 1993) or IgY (Davelaar *et al*., 1982), respiratory tract IgY (Holmes, 1973, 1979) in addition to serological IgA, IgM and IgY responses (Russell and Ezeifeka, 1995). The role of local antibodies in protection from respiratory disease is clearly important (Holmes, 1973), while the role of systemic antibody responses is potentially more important for the duration of immunity (Reynolds and Maraqa, 2000). In contrast, live IBDV vaccines, delivered in drinking water or *in ovo*, induce strong IgY virus-neutralizing responses in serum and, though this response is thought to correlate with protection against disease, an important role for CMI has also been postulated (Rautenschlein *et al*., 2002b).

Serum antibody responses are also induced with live salmonella and coccidiosis vaccines though local antibody responses are more likely to play some role in protection (Silva *et al*., 1981; Lillehoj and Lillehoj, 2000). In contrast no, or very weak, serum antibody responses have been detected following vaccination with live mycoplasma vaccines, again the local antibody response more likely to be playing a role in protection (Evans and Hafez, 1992).

The role of Th cells in the antibody responses against live vaccines has yet to be elucidated though it is known that production of IgY antibodies, normally requires help from antigen-specific $CD4^+$ Th cells (Arstila *et al*., 1994). In spite of the evidence for other correlates of protection, the systemic antibody response is used as a measure of immune status for many poultry vaccines, simple methods to study alternative potential IMCOP being unavailable.

Cell-Mediated Responses to Live Vaccines

Although antibody responses induced by live vaccines may play an important role in protection against some diseases, it is clear that CMI is an important mediator of protection against

intracellular pathogens. With the lack of tools to study CMI, bursectomies and the quantitation of T cell subsets following vaccination have been used to suggest that live vaccines mediate protection through non-humoral methods (Cook *et al*., 1991; Russell *et al*., 1997; Lillehoj, 1998; van Loon *et al*., 2003; Gimeno *et al*., 2004; Beal *et al*., 2006).

More recently, techniques allowing more precise analysis of the response induced by live vaccines have been used (Karaca *et al*., 2004; Degen *et al*., 2005b). Such mRNA detection techniques have allowed the demonstration of Th1 responses to virulent NDV (Degen *et al*., 2005b) and it is likely that live vaccines which replicate intracellularly will also induce Th1-polarized responses. Indeed, on the basis of IFN-γ production it can be suggested that use of live vaccines induces a Th1 type response to NDV, IBV, MDV, coccidia and salmonella. In addition, regardless of a good correlation between a virus-neutralizing antibody response and protection, live IBDV vaccines also induce a T cell response that could modulate IBDV pathogenesis (Rautenschlein *et al*., 2002b).

In spite of the difficulties associated with measuring CTL responses in chickens, reticuloendotheliosis virus (REV) (Maccubbin and Schierman, 1986), MDV (Omar and Schat, 1996), AIV (Seo *et al*., 2002) and IBV-specific (Seo and Collisson, 1997) CTL responses have been demonstrated. Furthermore, NDV-vaccinated birds depleted of B cells can resist NDV suggesting a role for CTL in NDV vaccinal immunity (Russell *et al*., 1997). The use of tetramers and analysis of peptides bound to the chicken MHC (Wallny *et al*., 2006) may help with the study of vaccine-induced CTL responses.

Immunosuppression and Interference of Live Vaccines

Although live vaccines have been shown to be very efficacious against a variety of poultry diseases, two consequences of vaccinating concurrently with several vaccines are immunosuppression and vaccine interference. Immunosuppression has been associated with the use of live MDV, IBDV, haemorrhagic enteritis virus and chicken infectious anaemia virus (CIAV) vaccines as these viruses replicate in cells associated with immune responses. In addition, vaccines that have similar tropisms are known to interfere with the immune response to each other (Cook *et al*., 2001; Ganapathy *et al*., 2005).

Responses to Inactivated Vaccines

When compared to replicating live vaccines, non-replicating antigens and especially the purified or recombinant subunit versions lack immunogenicity and are therefore mostly formulated in immunopotentiating vaccine adjuvants. Adjuvants are difficult to classify because they consist of a diverse group of molecules often with complex formulations. However, recently Schijns (2000, 2006) divided adjuvants into two groups (Table 20.2). The signal 1 facilitators influence the antigen location, residence or antigenic form, while the signal 2 facilitators directly activate the immune system either as non-self signals, derived from microbial origin ("stranger" signals; Janeway, 1992), or as inducers of endogenously liberated "danger" molecules (Matzinger, 1994). Recently, completely new signal 2 facilitating concepts have been proposed and demonstrated. These are based on inhibition by natural immune response inhibitors, including inhibition of suppressor of cytokine signalling-1 (SOCS1; Song *et al*., 2006), blockade of CTL-associated antigen (CTLA)-4 (Sutmuller *et al*., 2001), and depletion of Treg cells (Sutmuller *et al*., 2001; Jones *et al*., 2002).

Obviously, while the non-replicating antigen recognized by adaptive T and/or B cells determines the specificity of the immune response, the type of immunopotentiator will largely determine most other features of the immune response generated. These include onset, magnitude, duration, quality (Th1-, Th2- or Treg-associated), CMI and/or antibody dominated response, and the level of local and systemic reactions. In addition, the choice of immunopotentiator may preclude the need for a secondary immunization; hence reduce the amount of antigen needed. Moreover, some types of immunopotentiator may enable mucosal, needle-free delivery of the vaccine, or overcome non-responsiveness in elderly or immunologically immature neonatal hosts.

In many chicken vaccines, water-in-oil (W/O) emulsions are used to enhance efficacy and duration of immunity. The mechanism of action of such immunopotentiators is not exactly known, although in general W/O emulsions are believed to retain antigen at the injection site and thereby contribute to prolonged signal 1 delivery. Recently, Toll-like receptors (TLR)-2 involvement was speculated in mineral oil-based, W/O type formulations (Lim, 2003). We recently studied the structure–activity relationship of various W/O type formulations, formulated with identical ingredients. We observed that the type of oil strongly influences vaccine efficacy and also its safety (Jansen *et al*., 2005). For most inactivated vaccines W/O formulations induce antibodies extremely efficiently.

Optimal activation of innate immune reactions leading to migration and maturation of APC, including DC for primary responses, is essential for subsequent triggering of adaptive immunity (Degen *et al*., 2003). Innate immune reactions are activated within minutes, providing the first line of defence against invading pathogens, while adaptive immune cells require days before proper activation and clonal expansion. Innate immune cells express complement receptors, and so-called pattern recognition receptors, including TLR, mannose receptors, and scavenger receptors.

The recent discovery of TLRs on various innate immune cells acting as sensors for conserved microbial "stranger" molecules has vigorously revived the interest in innate immune reactions (see Chapter 7). Similar innate receptors are speculated to sense endogenous dangers molecules, e.g. heat shock proteins. This finding triggered an explosion of new information on this important phase of immune activation. In addition, many different TLR agonists, either as natural, purified products or as synthetic variants with increased safety profiles, are currently studied as candidate immunopotentiators with high potential for a variety of human and veterinary vaccines. For chicken vaccines most of these refined products may be too expensive, as yet. In addition, most TLR agonists require repeated administration which is currently not cost effective for the poultry industry. However, in chickens the homologies for TLR have recently been described (Roach *et al*., 2005; Chapter 7), and TLR ligands, such as CpG motifs, have been tested in a limited number of studies (Gomis *et al*., 2003).

Inactivated vaccines are most commonly used to vaccinate layer and breeder birds prior to the laying period. The inactivated vaccines may be used to boost immunity that has been provided by priming with live vaccines, for example, with IBV, NDV or IBDV vaccines. Alternatively, the inactivated vaccines are expected to provide life-long immunity without prior priming, for example, with egg drop syndrome (EDS) virus vaccines. The use of inactivated vaccines in breeders also confers immunity to progeny (maternal antibody, see Chapter 6), especially important for the control of IBDV infections. Whether used in primed or naïve animals, inactivated vaccines, especially those formulated in W/O adjuvants, induce high levels of serum IgY that can be maintained for over 1 year (Phillips, 1973).

Inactivated vaccines also induce T cell and inflammatory responses in chickens (Rautenschlein *et al*., 2002a; Okamura *et al*., 2004) though they are not as efficient at inducing local protection following aerosol challenge as live vaccines administered via a mucosal route (Beard and Easterday, 1967). However, it is thought that serum IgY can be transudated onto mucosal surfaces (Ewert *et al*., 1979) suggesting that inactivated vaccines can act locally. Indeed recent findings show that inactivated AIV vaccines formulated in a W/O adjuvant are able to limit the shedding of virulent H5N1 AIV from the respiratory and intestinal tract (Swayne *et al*., 2006) and have been successfully used in the face of an ongoing avian influenza outbreak in the field (Ellis *et al*., 2004).

CHICKEN VACCINE ADJUVANTS

Most vaccine adjuvants used for poultry include classical formulations, including W/O, oil-in-water (O/W), saponins and alum-based formulations. The exact mechanisms of such vaccine adjuvants remain undefined (Schijns, 2006). Adjuvants are currently used in many different registered

poultry vaccines and considered acceptable. The trend to include many distinct antigens in one formulation may hamper vaccine efficacy, as a result of interference between antigens in the final formulation or in the animal (V. Schijns and I. Tarpey, unpublished data).

STIMULATING MEMORY AND LONGEVITY OF IMMUNE RESPONSES

In naïve hosts, antigen-specific B and T cells exist at very low frequencies. After antigen-induced activation these cells start to proliferate and undergo clonal expansion, resulting in higher frequencies of antigen-specific effector B and T cells. After elimination of the antigen these effectors either die from apoptosis or go into a resting state resulting in very few surviving memory cells. Although the mechanisms for memory cell formation are poorly understood the strength and duration of antigen stimulation by APC are key to the priming of B and T cells (Iezzi *et al*., 1999; Lanzavecchia and Sallusto, 2002), and ultimately influence functional quality of memory cells (Seder and Ahmed, 2003). Overstimulation of T cells by repeated antigen exposure may tilt the balance towards terminal differentiation and T cell death (Jelley-Gibbs *et al*., 2005). Also antigen-independent factors affect memory cells, including certain cytokines, such as IFNs (Tough *et al*., 1999), and members of the common cytokine-receptor gamma-chain-cytokine family IL-7 and IL-15 (Becker *et al*., 2002; Schluns and Lefrancois, 2003; Bradley *et al*., 2005) and cellular interactions (Bachmann *et al*., 2004). For example, IL-15 is required for homeostatic proliferation to maintain populations of memory cells over long periods of time. One might reason that provision of exogenous cytokine or costimulatory molecule would boost memory formation after primary immune response induction. However, a booster immunization which contains antigens and which evokes sufficient endogenous costimulatory signals – if provided with the proper immunopotentiator or in case of a replicating attenuated strain – is more cost effective in most cases. Secondary immunization can be avoided, which is obviously preferred in poultry industry, when adequate stimuli are provided during primary immunization.

Although it has been demonstrated that a significant proliferation of immune cells takes place after vaccination, it remains unknown whether birds maintain their immune response in a similar manner to mammals. The stimulation of memory and duration of immunity of a vaccine is associated with the type of vaccine – live or killed (adjuvanted) – the delivery route, plus the age and immune status of the bird at vaccination. MDV vaccines, like many herpesviruses, are able to become latent (Holland *et al*., 1998), the recrudescence from which is likely to provide immune restimulation and concomitant life-long immunity. In contrast, avian metapneumovirus (aMPV), IBV and NDV vaccines that replicate in the respiratory tract for a maximum of 2 weeks have been shown to provide immunity for 12–14 weeks (Darbyshire and Peters, 1984; Cook *et al*., 1989) though immunity can begin to decline at 6 weeks (Gough and Alexander, 1979).

Differences in the duration of immunity are factors to be taken into consideration according to the type of bird being immunized. Broilers are usually kept for 6 weeks and a single vaccination generally suffices. In contrast, broiler breeders and layers may be kept for up to 2 years and it is unlikely that live vaccines (with the exception of MDV vaccines), especially if given at 1 day of age, can provide immunity for this period of time. For this reason, booster vaccinations are given usually in the form of inactivated vaccines. In most countries a boost to the antibody response that can maintain immunity to IBV, NDV, turkey rhinotracheitis virus (TRT) and IBDV throughout the laying period is essential. Little is known about the effect of boosting with inactivated adjuvanted vaccines following live priming, as only the antibody response has been measured. Regardless, this is an effective way to maintain the antibody response. It should be noted that vaccination with inactivated, adjuvanted vaccines such as those for EDS and salmonella, given between 12 and 18 weeks of age can protect birds throughout the laying period without the requirement for live priming. The adjuvants used in these vaccines may retain antigen at the injection site providing a prolonged signal 1 stimulus. A longitudinal study on the immune cell populations would greatly benefit our understanding of this subject.

DEVELOPMENT OF THE NEONATAL IMMUNE SYSTEM

In mammals neonatal B and T cells, when studied *in vitro*, show functional impairments relative to mature cell types. This may be explained by inherent, age-dependent cell-associated differences between the neonate's adaptive immune cells and their mature counterparts, or result from a different architectural context for B and T cell activation in the young (Fu and Chaplin, 1999). Inadequacies have been observed in detail at the level of B cell receptor signalling, often resulting in apoptosis and defective isotype switching (Burgnoni *et al*., 1994), and less pronounced expression of MHC class II and costimulatory molecules, resulting in diminished antigen presentation (Burgnoni *et al*., 1994; Tasker and Marshall-Clarke, 1997). Also, neonatal T cells show a bias towards Th2 type phenotype (Forsthuber *et al*., 1996) and are more susceptible to tolerance (Adkins, 1999). However, although somewhat impoverished under certain conditions, neonatal mammals are able to mount active immune responses if immunized with appropriate vaccines (Forsthuber *et al*., 1996; Ridge *et al*., 1996).

The development of the avian immune system begins early in embryogenesis. As described in Chapter 3, bursal precursor cells can be detected from embryonic incubation day (EID) 7 onwards and a rudimentary bursa, in which B cells later differentiate, from EID 10 onwards (Coltey *et al*., 1989; Mast and Goddeeris, 1999). Cells expressing surface IgM, IgY and IgA can be detected from EID 10, 14 and 16, respectively. Three distinct phases of B cell development have been observed (Houssaint *et al*., 1976; Arakawa *et al*., 1993; Paramithiotis and Ratcliffe, 1994; Chapter 4). Differentiation of T cells occurs in the thymus following the entry into this tissue of precursor cells during three closely regulated periods at EID 6.5, 12 and 18 (Coltey *et al*., 1989; Janse and Jeurissen, 1991; Chapter 3).

In accordance with the appearance of B and T cell progenitors in the embryo, adaptive immune responses to antigen may be inducible in chicken embryos from 12 to 14 days onwards (Solomon and Tucker, 1963; Solomon, 1966), though it should be noted that it is also possible to induce tolerance in embryos and newly-hatched chicks depending on the immunogen and the age of administration (Stevens *et al*., 1958; Hraba *et al*., 1982, 1984; Mast and Goddeeris, 1999; Zhang and Sharma, 2003). The vast majority of examples demonstrating a functional embryonic immune system have been with replicating micro-organisms. In particular, embryonic vaccination with MDV, IBDV and aMPV vaccines (Sharma, 1985; Worthington *et al*., 2003) at EID 18 has shown that the embryo can respond adequately to these viruses mounting protective immune responses at hatching. Vaccination *in ovo* with MDV vaccines has also demonstrated that the innate immune response is functional in embryos (Sharma *et al*., 1984). Interestingly embryo vaccination can result in better immune responses than post-hatching vaccination (Sharma and Burmester, 1982; Worthington *et al*., 2003). This could be linked to the fact that the vaccine can access parts of the embryo through oral and cloacal drinking of amniotic fluid, which are inaccessible to vaccines given after hatching. However, as the live vaccines replicate and persist, it is difficult to establish the immunocompetence of the embryo at the time of vaccination and in some regards the ability of the embryo to respond to a replicating organism may be linked with its ability to withstand the infection. This is clearly demonstrated by the fact that inoculation of 12- to 14-day-old embryos with herpesvirus of turkey (HVT), an MDV vaccine strain, can be lethal to the embryo (Zhang and Sharma, 2003), although at EID 18 the embryo can withstand, and respond to, vaccination with multiple micro-organisms (Sharma *et al*., 2002). The clearest evidence of a functional embryonic immune response has been demonstrated with vaccination of EID 16 embryos with inactivated non-adjuvanted *Campylobacter jejuni* (Noor *et al*., 1995). An antibody response with IgY, IgM and IgA isotypes was detected in serum, bile and intestinal scrapings of chicks 5 days after hatching. In addition, there was a significant increase in the number of Ig-containing cells in these tissues plus an increase of T cells in the blood.

Irrespective, it is clear that although embryos can mount innate and adaptive immune responses to vaccines, the response is sub-optimal in comparison to a mature immune system (Peters *et al*., 2003) with peak immunological maturity occurring several weeks after hatching (Sharma, 1997).

MATERNAL ANTIBODIES

Passive immunity provided by maternal antibodies was first demonstrated in 1893 by the transfer of immunity to tetanus toxin from vaccinated hens to chicks (Klemperer, 1893). Since then maternally-derived antibody (MDA) has become an important factor in deciding vaccination policy in the poultry industry. As described previously, prime boost strategies, using live vaccines followed by inactivated vaccines, are utilized to protect the laying hen or breeder from disease throughout the laying period. A consequence of this is the transfer of high levels of antibody into the progeny. Originally, there were doubts in regard to the isotypes that could be transferred to the yolk (Rose *et al*., 1974; Yamamoto *et al*., 1975), but is now generally accepted that IgY is exclusively transferred (Kowalczyk *et al*., 1985). IgY is sequestered across the follicular epithelium of the ovary into the egg yolk by a receptor-mediated transfer involving specific sequences found on IgY but not on IgM or IgA isotypes (Morrison *et al*., 2001). The amount of transfer of IgY is correlated with the amount of IgY present in the maternal circulation with transfer being delayed for approximately 5–6 days in chicken, and 7–8 days in turkeys. In the embryonated egg IgY is found in the egg yolk throughout the incubation period, in the egg white after EID 4, in the allantoic and amniotic fluids and embryo serum from EID 12 onwards. IgY in the yolk sac continues to be transferred for at least 48 h after hatching, so peak levels of MDA are not necessarily at 1 day of age. The half-life of the IgY in the serum of the hatched chick has been estimated to be between 3 and 6 days and in line with this MDA is catabolized over a period of 3–4 weeks in the progeny (see Chapter 6).

MDA is very protective especially when the antibody response correlates with protection. Chicks with MDA can be protected against infection with IBDV, but MDA also modulates infections with MDV, NDV, IBV and reovirus (Calnek and Smith, 1972; van der Heide *et al*., 1976; Darbyshire and Peters, 1985). In the case of IBV, IgY MDA can be detected in the respiratory mucus of hatched chicks (Hawkes *et al*., 1983). This antibody can protect chicks against IBV challenge (Darbyshire and Peters, 1985; Mockett *et al*., 1987), although since MDA in the respiratory tract declines more rapidly than serum antibody, a rapid decline in protection provided by MDA to respiratory diseases can result (Mondal and Naqi, 2001).

As MDA can modulate the growth of virulent pathogens, it also has considerable effects on the replication of live vaccines. IBDV vaccination policy is a classical example, whereby MDA can protect progeny chicks from virulent virus challenge for 3–4 weeks. This sets up a delicate balance with the need to have an active immune response to the live vaccine before a field challenge can infect the birds. Elaborate methods have been developed to measure the decline in IBDV-specific antibody in order to allow effective vaccination. In addition to IBDV, MDA has also been shown to affect the replication of MDV, IBV, NDV and reovirus vaccines though in the case of IBV, vaccination has been shown to reduce the levels of MDA. Prevention of the replication of vaccines suggests that MDA prevents the immune system from being exposed to the vaccines, a key factor in the maturation of the immune response.

IN OVO VACCINATION

In ovo vaccination is a relatively new procedure of mass vaccine delivery to poultry. In this procedure, certain live viral vaccines may be administered by injecting the inoculum in the egg during later stages of embryonal development. The vaccine is injected by inserting a needle into the broad end of the egg (Sharma and Burmester, 1982). The site of the deposition of the inoculum in the egg is somewhat variable and depends on the stage of embryonal development at the time of vaccination. Commercial egg-injection machines deliver the vaccine into the amniotic fluid of most eggs. The vaccine viruses do not adversely affect hatchability of eggs or performance of hatched chicks. Chicks hatching from vaccinated eggs show evidence of protective immunity at hatching (Sharma and Burmester, 1982). Following extensive laboratory testing in the 1980s (Sharma and Burmester, 1982; Sharma and Witter, 1983; Sharma *et al*., 1984; Sharma, 1985,

1986, 1987, 1989; Wakenell and Sharma, 1986), the *in ovo* vaccination technology was transferred to commercial broiler hatcheries (Ricks *et al*., 1999; Sharma, 1999). Currently, most of the major hatcheries in the USA and a number of other countries immunize broilers against MD by *in ovo* vaccination. In addition to the MDV vaccine, some flocks also receive *in ovo* vaccines against IBD and poxvirus. The *in ovo* vaccines are injected in eggs at about EID 18, when the eggs are routinely transferred from the incubators to the hatchers.

The successful transfer of the *in ovo* technology from the laboratory to the commercial hatcheries was facilitated by the development of automated multiple-head injector systems (MIS). The MIS are capable of simultaneously injecting vaccine inoculum into an entire tray of eggs. Under normal circumstances, two persons operating the MIS can vaccinate 30–50 000 eggs per hour. The ease of operating the MIS and the efficiency of processing large numbers of eggs account for the substantial savings in labour costs associated with conventional post-hatching vaccination procedures. The MIS are continually undergoing improvements. Newer models have incorporated technology that selectively injects vaccine only in eggs that contain viable embryos, thus minimizing vaccine wastage (Fig. 20.1).

The initial observations on *in ovo* vaccination were made by injecting serotype 3 MDV (HVT) in specific-pathogen-free eggs (Sharma and Burmester, 1982). The chicks hatching from vaccinated eggs were protected against challenge with virulent MDV at hatching. Subsequently, it was noted that embryos exposed to a mixture of related or unrelated live viruses responded immunologically to each component of the mixture (Sharma and Witter, 1983; Sharma, 1985; Sharma *et al*., 2002). A multivalent *in ovo* vaccine comprising five live viruses including serotypes 1, 2 and 3 MDV, an intermediate strain of IBDV and a recombinant fowlpox virus vector containing HN and F genes of NDV protected commercial broilers against virulent challenge with MDV, IBDV, NDV and fowlpox virus (Sharma *et al*., 2002).

Although current commercial applications of the *in ovo* vaccination technology are limited to the use of MDV, IBDV and fowlpox vaccines, a number of other agents including inactivated viruses and bacteria and DNA vaccines have been shown to induce an immune response following *in ovo* administration. Efforts are underway to initiate *in ovo* vaccination against

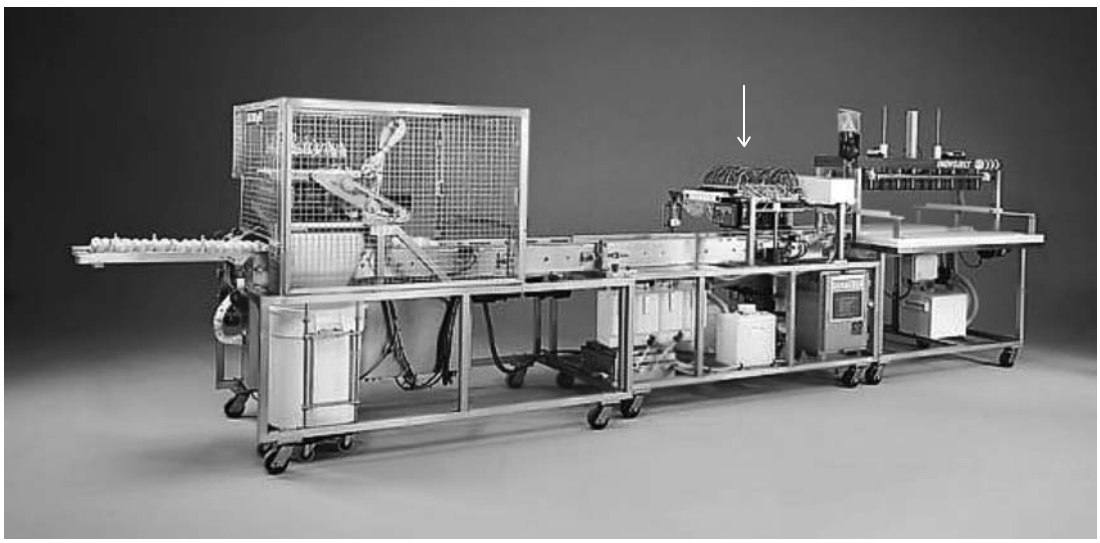

FIGURE 20.1 An *in ovo* vaccination machine with an optional candling and nonviable egg removal system used in commercial hatcheries. The tray of eggs first goes through an "egg remover" (left side of the figure) where infertile eggs and those with embryos that died within the first 10 days of incubation are removed. The tray containing eggs with mostly viable embryos moves to the right for vaccine injection by a multiple-head injector (arrow). Photo courtesy of Jason Fryar of Embrex, Inc.

coccidiosis using live oocysts. Table 20.3 summarizes the information on *in ovo* administration of some of the common poultry pathogens.

The mechanism by which *in ovo* administration of live viral vaccines results in protection against virulent agents in the hatched chick needs further study. Likely, the protection is

TABLE 20.3 Vaccine Candidates Examined for Possible *In Ovo* Administration

Vaccine	Current *In Ovo* Use	References
Marek's disease	Serotypes 1, 2 and 3 viruses used commercially	Sharma and Burmester (1982), Sharma and Graham (1982), Sharma and Witter (1983), Sarma *et al.* (1995), Gagic *et al.* (1999), Geerligs *et al.* (1999), Zhang and Sharma (2001), Sharma *et al.* (2002), Wakenell *et al.* (2002)
Infectious bursal disease	Mild and intermediate viruses and virus + antibody complex used commercially	Sharma (1985, 1986), Whitfill *et al.* (1995), Haddad *et al.* (1997), Jeurissen *et al.* (1998), Gagic *et al.* (1999), Coletti *et al.* (2001), Corley *et al.* (2001), Giambrone *et al.* (2001), Ivan *et al.* (2001), Corley and Giambrone (2002), Sharma *et al.* (2002), Negash *et al.* (2004)
Poxvirus	Limited commercial use of tissue culture origin fowlpox virus vaccines; fowlpox viruses also used as vectors for other poultry pathogens	Karaca *et al.* (1998), Gagic *et al.* (1999), Rautenschlein *et al.* (1999)
Infectious bronchitis	Attenuated virus protective under laboratory conditions	Wakenell and Sharma (1986), Borzemska *et al.* (1993), Wakenell *et al.* (1995)
Newcastle disease	Recombinants, viral mutants, killed virus and virus + antibody complex protective under laboratory conditions	Ahmad and Sharma (1992), Ahmad and Sharma (1993), Reddy *et al.* (1996), Stone *et al.* (1997), Karaca *et al.* (1998), Gagic *et al.* (1999), Rautenschlein *et al.* (1999), Ebrahimi *et al.* (2000), Mebatsion *et al.* (2001), Sharma *et al.* (2002), Mast *et al.* (2006)
Avian influenza	Experimental oil emulsion vaccine-induced immunity	Stone *et al.* (1997), Ebrahimi *et al.* (2000)
Reovirus	Reovirus alone or complexed with antibody: protective under laboratory conditions	Guo *et al.* (2003)
Hemorrhagic enteritis	Marble spleen virus of pheasants protective in turkeys under laboratory conditions	Fadly and Nazerian (1989), Ahmad and Sharma (1993)
aMPV	Attenuated virus protected turkeys under laboratory conditions	Worthington *et al.* (2003), Hess *et al.* (2004)
Adenovirus	Experimental use as a vaccine vector	Francois *et al.* (2004)
Protozoa	No protection against *Cryptosporidium baileyi*; good protection against coccidia using subunit and whole oocyst vaccines	Hornok *et al.* (2000), Ding *et al.* (2004), Dalloul *et al.* (2005), Lillehoj *et al.* (2005)
Bacteria	Experimental protection against *Campylobacter jejuni*	Noor *et al.* (1995)
Mycoplasma	Experimental use	El-Safty *et al.* (2005)
DNA vaccines	Successful immunization under laboratory conditions against coccidiosis, infectious bronchitis, infectious bursal disease and Newcastle disease	Oshop *et al.* (2002, 2003), Kapczynski *et al.* (2003), Lillehoj *et al.* (2005)

immunologically mediated. Both innate and adaptive immunity may be involved (Sharma *et al*., 1984; Sharma, 1989; Noor *et al*., 1995). As has been noted above, immune cells populate the embryo during early stages of embryogenesis. By about EID 14, the embryo acquires adequate functional capability to respond immunologically to an antigenic challenge (Solomon, 1966; Zhang and Sharma, 2003). Exposing embryos to live HVT at various stages of embryonation indicated that exposure at EID 14 or earlier resulted in immunological tolerance in 6–33% of the chickens. An active immune response rather than tolerance occurred when the viral exposure was delayed until the embryos were older than 14 EID (Zhang and Sharma, 2003).

At around 18 EID, the time when *in ovo* vaccines are administered, all functional components of the system are likely in place to mount a vigorous immune response. Further, live viruses deposited in the amniotic fluid readily access the embryonic target tissues for infection and replication (Sharma *et al*., 1984; Sharma, 1986, 1987; Ahmad and Sharma, 1993; Rautenschlein and Haase, 2005). Increased antigenic load in the host provides sustained immunological challenge as the embryo and the hatched chick becomes older and the immune system expands its functional capabilities. Certain non-replicating microbiological antigens may also persist in the embryo and provide substantial immunological challenge (Noor *et al*., 1995; Stone *et al*., 1997).

Advantages and Shortcomings of *In Ovo* Vaccination

Although *in ovo* vaccination has the advantage of inducing early post-hatch protection against MDV (Sharma and Burmester, 1982), in Europe and North America, the single most important reason for rapid commercial acceptance of the *in ovo* technology is the dramatic savings in the labour cost of administering MDV vaccines. The use of MIS allows simultaneous inoculation of an entire tray of eggs as opposed to subcutaneous injections in individual chickens required in the older methods. In addition, the MIS technology ensures the delivery of a uniform dose of the vaccine in each egg. In Asia and Latin America, where labour costs are not of major concern, improved vaccination efficiency and uniform vaccine delivery are the main reasons for increasing commercial acceptance of the *in ovo* vaccination technology. Laboratory and field studies have shown that multiple agents may be combined in the same vaccine inoculum, thus reducing the need for manipulating flocks for administering vaccines individually (Gagic *et al*., 1999; Sharma *et al*., 2002). The *in ovo* vaccination technology is being expanded to include non-vaccine related uses, e.g. chick sexing, delivering growth-promoting substances and *in ovo* feeding of nutrients to hasten intestinal maturity and improve feed conversion (Tako *et al*., 2005).

One of the shortcomings is that the *in ovo* technology is economically suitable only for large-volume hatcheries. Proper hatchery sanitation is critical to avoid losses due to environmental contamination of eggs being processed by the MIS. Also, the current use of *in ovo* vaccination is largely restricted to broiler flocks because eggs of both sexes need to be vaccinated. *In ovo* vaccination of breeder and laying flocks may gain popularity as gender-restricted MIS are developed for commercial use. Currently, *in ovo* vaccination is practiced only against a few selected diseases of chickens; suitable *in ovo* vaccines against some of the other important diseases including IBV and NDV are not yet available. The *in ovo* technology has not as yet been widely adopted by the turkey industry despite encouraging laboratory data on successful immunization against several common diseases.

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21 COMPARATIVE IMMUNOLOGY OF AGRICULTURAL BIRDS

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INTRODUCTION
INNATE IMMUNITY
CYTOKINES
CHEMOKINES
CELL SURFACE ANTIGENS
SURFACE IMMUNOGLOBULIN
MAJOR HISTOCOMPATIBILITY COMPLEX
SECRETED ANTIBODIES
CELL LINES
REFERENCES

INTRODUCTION

Although our knowledge of the immune system has advanced at a much faster pace for chickens than for other avian species, due to the sequence availability of the chicken genome, immunology of other agricultural birds has made great strides in recent years. Among these avian species, the immune system of the duck has been studied most extensively. Thus, much of the information presented here is based on studies using ducks. A strong interest in the phenotypic characterization of duck immune cells and immunoregulatory mediators such as cytokines and chemokines arose from its role as surrogate infection model for the hepatitis B virus (HBV), the causative agent of acute and chronic hepatitis B in man. Pekin ducks (*Anas platyrhynchos*) are the natural host of a related hepadnavirus, duck hepatitis B virus (DHBV) (Mason *et al.*, 1980). Like HBV, DHBV has a narrow host-range, such that it does not infect Muscovy ducks (*Cairina moschata*), although they belong to the same order as Pekin ducks (Marion, 1988; Pugh and Simmons, 1994). Noteworthy, it is not the virus *per se* that causes the death of infected hepatocytes in man but rather HBV-specific cytotoxic T cells are involved in destruction of the liver. Although infection in the duck exhibits the same age-related outcomes (Jilbert *et al.*, 1992; Jilbert *et al.*, 1998), there is no histological evidence that DHBV is associated with hepatitis in ducks.

More recently, researchers investigating highly pathogenic influenza A viruses became interested in the regulation of immune responses in ducks and other domesticated agricultural avian species. Ducks play a key role in the maintenance and dispersal of influenza A viruses, serving as the natural reservoir. All strains of influenza viruses have been isolated from waterfowl (Webster *et al.*, 1992). Ducks are usually asymptomatic carriers of influenza viruses, and

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these can be transmitted to other avian and mammalian hosts, including humans. The recent H5N1 avian influenza outbreaks have caused mortality in ducks, although a range of pathogenicity is seen with different isolates (Sturm-Ramirez *et al.*, 2005). Ducks are implicated in the propagation and endemnicity of this virus, contributing to its spread throughout Asia and Europe. Development of vaccines for poultry (including ducks) is underway and their evaluation depends on having measurable parameters for avian immune responses (Tian *et al.*, 2005; Webster *et al.*, 2006).

In Europe and many other Western countries, the consumption of bird meat, other than chicken meat, is increasing annually. In view of the growing number of commercially held turkeys (*Meleagris gallopavo*), ducks (*A. platyrhynchos* and *C. moschata*), and geese (*Anser anser*) and their economic importance, these birds need to be protected against pathogens by improving their natural and specific defence mechanisms, for instance by vaccination. Therefore, approaches improving our understanding of the immune systems of these species are most desirable.

Domestic production of the ostrich (*Struthio camelus*) in countries other than South Africa started in the 1970s. Production spread quickly and, today, ostrich are raised in many countries including: USA, Australia, Canada, Europe, China and Korea. Producers today are breeding ostrich mainly for their meat, with the hides, feathers, fat, egg shells and other parts of the birds providing valuable by-products.

Here, we review the immune system of turkeys, quails, ducks, geese and ostriches with an emphasis on recent studies concerning the molecular characterization of cytokines, chemokines and cell surface markers.

INNATE IMMUNITY

Toll-Like Receptors

Innate immunity provides a first line of host defence against infection through microbial recognition and killing while simultaneously activating a definitive adaptive immune response (see Chapter 7). Toll-like receptors (TLR) belong to a multigene family that help to detect foreign invaders by sensing various pathogen-associated molecular patterns which have been conserved in both the invertebrate and vertebrate lineages. Binding of ligands to TLR activate signal transduction pathways and induce the expression of a wide variety of host defence genes, such as antimicrobial peptides, cytokines, chemokines and nitric oxide synthase (Barton and Medzhitov, 2002). TLR genes have been recognized in a number of vertebrate genomes, such as those of mouse, man, chicken, fish (rainbow trout *Oncorhynchus mykiss*, Japanese flounder *Paralichthys olivaceus*, goldfish *Carassius auratus*) and amphibia (frog *Xenopus tropicalis*). There are six major families of vertebrate TLR which are characterized by the recognition of a general class of pathogen-associated molecular patterns. Members of the TLR families are described in detail in Chapter 7.

The chicken genome possesses genes for TLR1/6 (type 1 and 2), TLR2 (type 1 and 2), Type 3-5, TLR7, TLR15 and TLR21 (Lynn *et al.*, 2004; Smith *et al.*, 2004; Philbin *et al.*, 2005; Yilmaz *et al.*, 2005). It has been suggested that TLR15 may represent an avian-specific TLR that has been either retained in chicken and lost in other taxa or gained in the chicken (Higgs *et al.*, 2006). Recent characterization of the chicken TLR7/8 loci revealed an intact TLR7 gene and fragments of a TLR8-like gene with a 6-kilobase insertion containing a chicken retroviral-like insertion element (CR1) (Philbin *et al.*, 2005). Other galliform species have also been shown to be positive for this insertion element, including Japanese quail, guinea fowl, pheasant and turkey. The size of the genomic PCR product is the same in galliform species except for Japanese quail where it is shorter. Interestingly, there was no evidence for a CR1-TLR8 disruption in non-galliform birds, including Pekin duck, goose, turkey and ostrich. However, there is no information on whether these species actually possess TLR8-specific sequences in their genomes. In a parallel experiment using the same DNA samples it was possible to amplify

TLR7-specific sequences from all the species tested, indicating that TLR7 is widely expressed within the order *Aves*. To examine this question in ducks, we have isolated and sequenced a genomic clone encoding the TLR7 locus, and find no evidence of an intact TLR8 gene (M. MacDonald, A. Smith and K. Magor, unpublished data). Recent database entries provide sequence information on TLR7 (acc. no. AY940195) and TLR2 (acc. no. AY838880) of Pekin ducks (G.Y. Reaiche and A.R Jilbert, unpublished data). The cDNA fragments contain sequence information on the membrane proximal region of the Toll interleukin 1-like receptor (TIR) domains which show a high degree of identity on the nucleotide level to the respective chicken cDNA fragments (~90%). No avian TLR9 homologue has been identified. However, chickens are capable of responding to unmethylated bacterial CpG DNA, the ligand of mammalian TLR9 (He *et al.*, 2003; Xie *et al.*, 2003). TLR7 may be the candidate receptor for recognition of these molecules in chicken, because the structure and location of leucine-rich repeats are similar to human TLR9. Due to the lack of information on TLR of other avian species it is not known whether TLR9 is absent from genomes of other domesticated agricultural birds.

Antimicrobial Peptides

Antimicrobial peptides are considered endogenous antibiotics that play a multifunctional role in innate immunity of mammalian and avian species. Among these, β-defensins contribute to mucosal and epithelial defence, also acting as signal molecules for cellular components of innate and adaptive immunity (see Chapter 7). Avian β-defensins have been found in blood of chickens, turkeys and ostriches; epithelial cells of chickens and turkeys and stomach contents of king penguins. Thirteen different members of this family have been identified in chickens (Gal1-13) and no other groups of defensins could be identified in the chicken genome in a comprehensive screen (Xiao *et al.*, 2004). In addition, three β-defensins have been isolated from turkey heterophils (turkey heterophil peptide, THP-1 to -3) so far. THP-1 killed *Escherichia coli* and *Staphylococcus aureus in vitro*, whereas THP-2 and THP-3 killed only *Staph. aureus* (Evans *et al.*, 1994). Four β-defensins, named ostricacins (OSP) 1-4, have been purified from ostrich heterophils and characterized (Yu *et al.*, 2001; Sugiarto and Yu, 2006). Comparison with the known β-defensins from mammals and chickens revealed that the four ostricacins share the six conserved cysteine residues known as the "β-defensin motif". The ostricacins were effective in inhibiting the growth of Gram-positive and Gram-negative bacteria, but as chicken, turkey and king penguin β-defensins, ostricacins displayed only moderate activity against yeast. So far an antimicrobial activity against filamentous fungi has been described only for king penguin β-defensin (Thouzeau *et al.*, 2003). Furthermore, a GenBank search revealed the sequence of a duck cDNA encoding a putative β-defensin (acc. no. AY641439, E. Sreekumar, A. Premraj and T.J. Rasool, unpublished data). The cDNA has been generated from RNA isolated from duck spleen cells. Amino acid sequence comparison revealed the closest sequence identity with OSP-1, Gal-2 and THP-2 (75–78%). A second β-defensin (acc. no. DR764075) was identified through a duck spleen expressed sequence tag (EST) project (Xia *et al.*, 2007) and further characterized as most similar to THP-1 of turkeys (A. Oko and K. Magor, unpublished data). The identification of β-defensins in ducks supports the idea that avian antimicrobial peptides are mainly composed of β-defensins. Further work is required to study other roles of avian β-defensins in innate and adaptive immunity such as the chemotactic function shown by their mammalian counterparts.

CYTOKINES

Interferons

Type I Interferon

The first cDNA for an avian type I interferon (IFN) was identified by Sekellick *et al.* (1994) by an RT-PCR approach that employed degenerate primers derived from conserved regions

of mammalian IFN-α and IFN-β genes on RNA isolated from virus-induced chicken embryo cells. The poor degree of sequence identity to mammalian IFN-α and IFN-β (<25% identity on the amino acid level) probably explains the long time it took to progress from the "interfering factor" identified by Isaacs and Lindenmann (1957) in allantoic membranes of embryonated chicken eggs to the molecular clone. Thereafter, an additional intronless chicken type I IFN gene was identified whose primary translation product was only 57% identical with the previously cloned chicken type I IFN. Functional studies indicate that the two type I IFN represent the homologues of mammalian IFN-α and IFN-β (Sick *et al.*, 1996, 1998). The cloning of the two chicken IFN set the ball rolling. Subsequently, employing homology screening approaches IFN-α genes of other birds have been cloned. Identity to chicken IFN-α at the amino acid level varies: 82% in turkey (Suresh *et al.*, 1995), 51% in duck (Schultz *et al.*, 1995) and 48% in goose (Li *et al.*, 2006; Table 21.1). Despite the low amino acid sequence identity between chicken (ch)IFN-α and duck (du)IFN-α, duIFN-α exhibited an antiviral activity on CEC-32 cells (see cell lines), though the effect was ~12-fold less than on duck cells. In contrast, chicken IFN-α, which exhibited a high antiviral activity on CEC-32 cells, exhibited no detectable activity on duck cells (Schultz and Chisari, 1999). Comparison of the antiviral activity of chicken IFN-α with turkey IFN-α revealed that chicken IFN-α reacted to a similar extent with both chicken and turkey cells, while turkey IFN-α exhibited a 16-fold lower activity on chicken cells (Suresh *et al.*, 1995). Single cell analysis revealed the existence of 10 copies of closely related intronless IFN-α in ducks (Zhang *et al.*, 2003; U. Schultz, unpublished). As in chickens they form a cluster on the short arm of the duck Z chromosome (Nanda *et al.*, 1998). Recombinant duck IFN-α has been shown to effectively inhibit DHBV replication in primary hepatocyte cultures and in ducklings (Schultz *et al.*, 1995; Heuss *et al.*, 1998; Protzer *et al.*, 1999). In addition, the two steps of the DHBV replication cycle which are sensitive to IFN could be identified (Schultz *et al.*, 1999). No IFN-β genes of birds, other than that of chicken, have been described to date. Interestingly, the level of amino acid sequence identity of duck IFN-α to chicken IFN-α (51%) and chicken IFN-β (52%) is virtually identical. However, like chicken IFN-α, the cloned duck IFN is a member of a multigene family, and is strongly expressed in response to virus infection and oral treatment with imidazoquinoline S-28463 (ligand for TLR7). These results support the view that the cloned duck IFN represents the functional homologue of mammalian IFN-α (Schultz *et al.*, 1999). There is no information on type I IFN receptors in birds other than for chicken. However, there are several studies involving quail and duck cells that describe the existence of factors known to be involved in the signalling pathway of type I IFNs (Heuss *et al.*, 1998; Zoller *et al.*, 2000) as well as of genes known to be induced by type I IFNs (Schultz and Chisari, 1999). Recently, components of the IFN regulatory pathway have been identified in our duck EST project, including STAT1 (acc. no. DR766257), IRF-4 (acc. no. DR764421), IRF-8/ICSBP (acc. no. DQ266446) and an IFN-stimulated gene ISG12 (acc. no. DR764528) (J.C. Xia, X.G. Radford and K. Magor, unpublished).

Type II Interferon

Special attempts were undertaken to identify IFN-γ genes in domesticated agricultural birds with the aim of using this cytokine to influence the immune response to pathogens in these species that are of growing economic importance. In addition, to be able to investigate the role of IFN-γ in viral clearance during a natural HBV infection in the duck, it seemed to be most desirable to clone the respective gene of ducks. Functional approaches were employed to identify cDNA encoding chIFN-γ (Digby and Lowenthal, 1995; Weining *et al.*, 1996). Subsequently, cDNA from several other galliform species, including Japanese quail and turkey, were cloned by RT-PCR using degenerated primers designed to the chIFN-γ sequence (Kaiser *et al.*, 1998; Loa *et al.*, 2001; Table 21.1). The level of identity within the galliform species was determined to be 93.9–97.6%. As suggested by the high amino acid sequence identity among these species, both turkey IFN-γ and chicken IFN-γ could induce production of nitric oxide by both turkey and chicken macrophages. This activity could be neutralized by a monoclonal antibody (mAb) raised against chicken IFN-γ indicating a close antigenic relationship (Lawson *et al.*,

TABLE 21.1 List of Cloned Duck, Goose, Turkey and Quail Cytokine cDNA

Cytokine	Accession Number	References
Duck[a]		
• Interferon		
IFN-α	X84764; AB128861; AY879230	Schultz *et al.* (1995), Xia *et al.* (2000), B. Chen, A. Cheng, M. Wang and Q. Tang, unpublished
IFN-γ	AF087134; AJ012254; AY166850	Schultz and Chisari (1999), Huang *et al.* (2001), Wu *et al.* (2001)
• Interleukin		
IL-1β	AY426338[b]	Humphrey *et al.*, 2004
IL-2	AF294322, AF294323; AY232490, AY707747, AY173028, AY821656, AY193713[c]	K. Schmohl and U. Schultz, unpublished; Sreekumar *et al.* (2005b), Zhou *et al.* (2005b)
IL-6	AB191038[b]	X.F. Ruan and C. Xia, unpublished
IL-15	DQ490136	U. Schultz, unpublished
IL-16	AF294320, AF294321	K. Schmohl and U. Schultz, unpublished
IL-18	AF336122; DQ490137	W.-S. Chan, G.W. Warr, D.L. Middleton, M.L. Lundqvist and D.A. Higgins, unpublished; N.K. Mannes and U. Schultz, unpublished
• Chemokine		
CC chemokine "MIP-1β"	AY641437	Sreekumar *et al.* (2005a)
CC chemokine "RANTES"	AY641435	Sreekumar *et al.* (2005a)
CC chemokine	AY641436	Sreekumar *et al.* (2005a)
CCL19	DR766004	Xia *et al.* (2007)
CCL21	DR764376	Xia *et al.* (2007)
CXC chemokine "chCXCLi2/IL-8-like"	AB205199[b]	W.H. Chen and C. Xia, unpublished
• Other cytokines		
CD154	DQ267671	Fischer *et al.* (2007)
BAFF	DQ445092	Guan *et al.* (2007)
Goose		
• Interferon		
IFN-α	AY524422[d]	Li *et al.* (2006)
IFN-γ	AY524421[d]	J. Mi, J. Wang, L. Xu and H. Li, unpublished
• Interleukin		
IL-2	AY392557[e]	Zhou *et al.* (2005a)
• Chemokine		
CXC chemokine "chCXCLi2/IL-8-like"	AB213393[e]	R.L. Guan, W.H. Chen and C. Xia, unpublished
Turkey		
• Interferon		
IFN-α	U28140	Suresh *et al.* (1995)
IFN-γ	AJ000725	Kaiser *et al.* (1998)
• Interleukin		
IL-2	AJ007463; AF209705	Lawson *et al.* (2000), C.H. Romero and X.Z. Cai, unpublished
IL-12β-chain	AJ564203[b]	Balu and Kaiser (2003)
IL-18	AJ312000	Kaiser (2002)
Quail		
• Interferon		
IFN-α	AB154298	K. Koyama and T. Takahashi, unpublished
IFN-γ	AJ001678	Kaiser *et al.* (1998)
• Interleukin		
IL-2	AY707748	Sreekumar *et al.* (2005b)

[a]Pekin duck (*Anas platyrhynchos*) except of sequences indicated.
[b]Partial coding sequence.
[c]Muscovy duck (*Cairina moschata*).
[d]"Grey" goose (*Anser anser*).
[e]Swan goose (*Anser cygnoides*).

2001; Loa *et al.*, 2001). Using the chIFN-γ cDNA as probe, duIFN-γ was identified in lambda phage cDNA libraries of mitogen-stimulated splenocytes (Schultz and Chisari, 1999; Huang *et al.*, 2001; Table 21.1). In addition, a recent database entry provides sequence information on goose IFN-γ (acc. no. AY524421, J. Mi, J. Wang, L. Xu and H. Li, unpublished). The amino acid sequence identity within the anseriform species was determined to be 93.3%. Both duck and goose IFN-γ show a lower degree of sequence identity to galliform IFN-γ (67–68%). Nevertheless, duIFN-γ showed antiviral activity on CEC-32 cells, though the effect was ~16-fold less than on duck fibroblasts. It had also been reported that recombinant duIFN-γ had the capacity to induce chicken macrophages to produce nitric oxide. Again, the specific activity was approximately 16-fold lower than that detected with chIFN-γ. Further indication of similarity among these two avian IFN-γ was obtained by the demonstration that a rabbit serum raised against duIFN-γ could neutralize the activity of chIFN-γ (Huang *et al.*, 2001). In contrast, chIFN-γ like chIFN-α exhibited species-specific activity in that it showed undetectable levels of reactivity with duck fibroblasts. Importantly, duIFN-γ blocked the replication of DHBV in primary duck hepatocytes (Schultz and Chisari, 1999).

Interleukins

Interleukin-1

Isolation of a cDNA encoding chicken interleukin (IL)-1β was achieved by expression cloning using a cDNA library generated from lipopolysaccharide (LPS)-stimulated HD-11 cells (Weining *et al.*, 1998). Subsequently, a 637 base pair cDNA fragment could be amplified by PCR from Pekin duck liver using primers designed to the chIL-1β cDNA. The amplicon had 85% homology at the nucleotide level with the chIL-1β mRNA sequence (Humphrey *et al.*, 2004; Table 21.1). The cDNA sequence, as deposited in GenBank, did not contain a continuous open reading frame (ORF) and lacked part of the N- and C-terminal coding sequence. However, the region of homology included the mammalian cleavage site for IL-1β converting enzyme. Resembling chIL-1β the predicted amino acid sequence of duIL-1β lacked the highly conserved aspartic acid residue necessary for recognition and cleavage by the protease. It could be shown that IL-1β is expressed in spleen and liver of ducks in response to an intravenous injection of *E. coli* complexed to various ratios of immunoglobulin (Ig)Y:IgY(ΔFc). While splenic duIL-1β expression decreased sharply as the amount of IgY(ΔFc) in the immune complexes (IC) increased, induction of hepatic IL-1β expression by IC was not correlated to the IgY:IgY(ΔFc) ratio. The authors assumed that the effects of IgY:IgY(ΔFc) ratios on splenic IL-1β mRNA expression are due to this organs dependence on C to facilitate IC uptake. Thus, Kupffer cells in the liver seem to possess receptors that recognize IgY(ΔFc)-antigen complexes as well as IC comprising full length IgY (Humphrey *et al.*, 2004).

A cDNA for the IL-1 receptor chain 1 (IL-1R1) of chicken has been cloned (Guida *et al.*, 1992). cDNA for IL-1 β and IL-1R homologues in other avian species have not been identified to date. Furthermore, cDNA for the avian homologue of IL-1α awaits identification.

Interleukin-2

The first avian IL-2 to be cloned was that of chicken. It was achieved by expression cloning, testing supernatants of cells transfected with pools of cDNA for their proliferation-inducing activity (Sundick and Gill-Dixon, 1997). In earlier studies duck lymphocytes stimulated with phytohaemagglutinin (PHA) have been shown to release cytokines, which could induce the proliferation of duck lymphoblasts (Higgins *et al.*, 1993; Bertram *et al.*, 1997). Using the chIL-2 cDNA as probe, two Pekin duIL-2 cDNA were identified by screening a lambda phage cDNA library from PHA-stimulated duck splenocytes. The two duIL-2 cDNAs have 423 bp long ORF coding for identical proteins. However, the 3′ UTR of the two clones vary in length by 63 nucleotides. One cDNA was found to have four repeats of the instability motif ATTTA in the 3′ UTR while the other has six of these repeats (K. Schmohl and U. Schultz, unpublished; Table 21.1).

Subsequently, IL-2 cDNA of Muscovy duck (Zhou *et al.*, 2005b), goose (Zhou *et al.*, 2005a), turkey (Lawson *et al.*, 2000; AF209705, C.H. Romero and X.Z. Cai, unpublished) and Japanese quail (Sreekumar *et al.*, 2005b) were identified by RT-PCR using sequence information on the previously identified chIL-2 and duIL-2 (Table 21.1). The cDNA contain complete ORF encoding proteins of 140–143 amino acids. Analysis of the gene organization revealed remarkable conservation between avian and mammalian IL-2 genes, with four exons and three introns and a very short 5′ UTR. Some variations were observed in the length of the third intron, which was of similar size in chicken and quail, but was slightly longer in Pekin duck. Transcription factor binding sites and the predicted transcription start site with respect to the chIL-2 sequence showed total conservation in Pekin duck, quail and turkey (tu)IL-2 promoters (Lawson *et al.*, 2000; Sreekumar *et al.*, 2005b). When the homologous region of chicken and duck IL-2 promoter sequences were compared a sequence identity of 88.8% was observed, whereas between chicken and quail and chicken and turkey identities of 97% and 95.7%, respectively, were observed (Lawson *et al.*, 2000; Sreekumar *et al.*, 2005b). Comparative analysis of avian IL-2 amino acid sequences revealed identities varying from 59.3% (chicken:Muscovy duck) to 95.7% (Pekin duck:Muscovy duck). Phylogenetic analysis of avian IL-2 amino acid sequences shows that the similarity of avian IL-2 sequences parallels the presumed evolutionary relationships between the species: the galliform birds (chicken, turkey, quail) forming one clade and the anseriform birds (Pekin duck, Muscovy duck, goose) forming a distinct clade. A fragment corresponding to mature duIL-2 was expressed in 293T cells (K. Schmohl and U. Schultz, unpublished) and in *E. coli* (Zhou *et al.*, 2005b) and was shown to be biologically active in lymphocyte proliferation assays. Interestingly, duIL-2 and chIL-2 cross-react in lymphocyte proliferation assays although they share only limited sequence identity (Zhou *et al.*, 2005b). tuIL-2 and chIL-2 (~70% identity) have also been shown to cross-react in functional assays (Lawson *et al.*, 2000). Recombinant goose IL-2 induces goose, duck and chicken lymphocytes to proliferate, though the observed effect on duck and chicken lymphocytes has been relatively weak (~83% and 63% amino acid sequence identity, respectively; Zhou *et al.*, 2005a).

Furthermore *in vivo* bioactivity of recombinant duIL-2 and goose IL-2 was assessed in vaccination studies (Zhou *et al.*, 2005a, b). Recombinant IL-2 strengthens duck and goose immune responses, respectively, induced by inoculating an inactivated vaccine against avian influenza virus (rise in virus-specific antibody titres). Polyclonal antibodies and mouse mAb raised against *E. coli*-produced duIL-2 and goose IL-2 were shown to be able to neutralize the biological activity of both recombinant and endogenous duIL-2 and goose IL-2, respectively (Zhou *et al.*, 2005a, b). Both the recombinant duIL-2 and the respective mAb will be valuable tools for future work aimed at elucidating the effect of cytokines on the outcome of an HBV infection in the duck and immunological studies on the avian immune system in general. No functional data are available for quail IL-2 at present.

IL-2 and IL-15 are structurally related cytokines. The receptor of both cytokines is composed of three subunits (IL-2Rα/CD25, IL-2Rβ and IL-2Rγ). While both cytokines have their own α-chain they share the β- and the common γ-chain. Information on avian homologues is few and far between. A cDNA clone for an α-chain of the putative chicken IL-2 receptor can be found in the database (acc. no. AF143806, H.S. Lillehoj, H.B. Kim, K.D. Choi, K.D. Song, W.G. Min and J. Burnside, unpublished) as well as several EST clones similar to the mammalian IL-2Rγ-chain (Carre *et al.*, 2006). A recent database entry provides sequence information on the α-chain of the duIL-2R. The cDNA contains an ORF of 633 nucleotides that gives rise to a primary translation product of 211 amino acid residues which shows 60.7% and 24.6% identity to chIL-2Rα and human IL-2Rα, respectively (acc. no. DQ299949, Wang *et al.,* 2007). An EST encoding a partial duIL-2Rγ-chain (acc. no. DR763813) showed 87% amino acid identity to the chicken sequence (Xia *et al.*, 2007).

Interleukin-6

In the duck, an IL-6-like biological activity was first demonstrated in supernatants of LPS-stimulated duck monocytes (Higgins *et al.*, 1993). While supernatants of LPS-stimulated

monocytes induced proliferation of the murine cell line 7TD1, this activity could not be observed in supernatants of mock-treated cells. However, no IL-6-specific transcripts could be detected by northern blot in RNA preparations from lymphoid organs of normal and antigen-stimulated ducks when hybridized with a human IL-6 cDNA probe (Higgins *et al.*, 1993). Meanwhile the sequence of a cDNA covering 383 bp of a putative duIL-6 ORF has been deposited in GenBank (acc. no. AB191038, X.F. Ruan and C. Xia, unpublished; Table 21.1). The nucleotide sequence translates into a protein that shows high amino acid sequence identity with chIL-6 (89.9%) and only 45.7% identity was observed with human IL-6.

Interleukin-8

In mammals IL-8 is a CXC chemokine and has therefore been renamed CXCL8. Molecules that may represent the avian homologue of mammalian IL-8/CXCL8 are dealt with in the section on chemokines (Table 21.1).

Interleukin-12

In mammals, IL-12 is a heterodimeric 70 kDa cytokine composed of two components, a 40-kDa heavy chain (IL-12β or p40) and a 35-kDa light chain (IL-12α or p35). Both chains from chicken have been cloned taking advantage of partial chicken EST sequences with homologies to mammalian IL-12 subunits (Balu and Kaiser, 2003; Degen *et al.*, 2004).

After the initial discovery of chIL-12p40 a partial turkey IL-12p40 cDNA was obtained by RT-PCR from turkey splenocyte RNA using primers designed to the chIL-12p40 cDNA sequence. The turkey cDNA encodes a predicted protein with 95% identity to chIL-12p40 (Balu and Kaiser, 2003; Table 21.1). To our knowledge no further IL-12 genes have been identified for domesticated agricultural birds.

Interleukin-15

To date the cloning of IL-15 cDNA have been described only in two avian species, chicken and duck. The cDNA for duIL-15 was identified by screening a lambda phage cDNA library from PHA-stimulated duck spleen cells with the chIL-15 cDNA probe (U. Schultz, unpublished data; Table 21.1). The 876-bp duIL-15 cDNA consists of an ORF coding for a predicted protein of 182 amino acids. With 66 amino acids the signal peptides of both duIL-15 and chIL-15 are longer than that of the human counterpart. The predicted mature duIL-15 showed ~76% identity with chIL-15 and only ~30% identity with human IL-15. No functional data are available at present.

Interleukin-16

The identification of a complete duIL-16 cDNA was achieved by screening a lambda phage cDNA library from PHA-stimulated duck spleen cells (K. Schmohl and U. Schultz, unpublished). A cDNA that contained information on the C-terminal moiety of chIL-16 was used as a probe. Two clones that differed in the size of the cDNA (2070 bp and 2340 bp) have been chosen for further evaluation (acc. no. AF294320, AF294321; Table 21.1). Both cDNA contained an ORF comprising 1821 bp and gave rise to proteins differing only in 3 amino acid residues with molecular weights of 65 kDa. Amino acid sequence comparison of the duIL-16 propeptide with the propeptide of chIL-16 and human IL-16 indicated that duIL-16 shares 86% identity with chIL-18 and 51% with human IL-16. In addition, it revealed the presence of four aspartate residues (477, 486, 495, 499) in a region in which cleavage of mammalian propeptides by caspase 3 occurs. Aspartate 486 of the duIL-16 propeptide is closest to the aspartate residue, which is used for recognition and cleavage of human IL-16 by the protease, however this aspartate residue is not conserved in chIL-16. duIL-16 transcripts were expressed abundantly in lymphoid tissues (K. Schmohl and U. Schultz, unpublished). No functional data are available at present.

Interleukin-18

At present IL-18 cDNAs from three avian species have been cloned: chicken (Schneider *et al.*, 2000), turkey and duck. The cDNA of tuIL-18 was cloned by RT-PCR from PMA-stimulated

turkey splenocytes using oligonucleotide primers based on the sequence of the chIL-18 cDNA (Kaiser, 2002; Table 21.1). Subsequently, sequences flanking the coding sequences were isolated by 5′ and 3′ RACE. While the 3′ UTR of chIL-18 has only one copy of the instability motif ATTTA the tuIL-18 3′ UTR contains two copies of this motif, which is involved in rapid mRNA degradation. Overall the deduced tuIL-18 amino acid sequence has 97.4% identity with the chIL-18 sequence. No information is available to date on the biological activity of tuIL-18. Two database entries provide sequence information on IL-18 of ducks (Table 21.1). The sequence deposited by Higgins and coworkers consists of 705 nucleotides and encodes for a protein of 200 amino acids (acc. no. AF336122, W.-S. Chan, G.W. Warr, D.L. Middleton, M.L. Lundqvist and D.A. Higgins, unpublished). Duck thymus RNA was used to amplify duIL-18 cDNA, from the start of the precursor molecule to the stop codon, using primers of the duIL-18 cDNA meanwhile deposited by Higgins (acc. no. DQ49013, N.K. Mannes and U. Schultz, unpublished). Comparison of the two deduced amino acid sequences revealed 4 amino acid differences (V11E, L19P, F21Y, Q54R). They showed ~86% identity to chicken and turkey IL-18, 26.4% to human IL-18 and 20% or less to fish IL-18. Like chicken, turkey and mammalian IL-18, bioactive duIL-18 seems to be generated from an inactive precursor by cleavage at a conserved aspartate residue after amino acid residue 30 to give rise to a mature protein with a calculated molecular mass of 19.7kDa. Recombinant duIL-18 produced in *E. coli* induces the proliferation of duck splenocytes and the secretion of IFN-γ from the chicken B cell line B19-2D8 indicating that duIL-18 is able to bind to and activate the chicken IL-18 receptor. The IFN-γ-inducing activity could be neutralized by preincubation of duIL-18 with a polyclonal rabbit antiserum raised against duIL-18. IL-18 gene expression has been observed in peripheral blood lymphocytes (PBL), spleen, thymus and bursa of Fabricius but not in liver (N.K. Mannes and U. Schultz, unpublished).

Tumour Necrosis Factor Family

The tumour necrosis factor (TNF) superfamily consists of members that are distantly related to TNF and have important functions in the regulation of immune responses, inflammation and tissue homeostasis. Very few members have been characterized in birds at present and although various biological activities have been described for supernatants of stimulated chicken macrophages believed to contain the chicken homologue of TNF-α, to our knowledge, nucleic acids encoding *bona fide* TNF-α have not been identified in avian species to date. Chicken B cell activating factor (BAFF) has been cloned after a cDNA was identified in a chicken bursa EST database. Soluble recombinant chicken BAFF has been shown to bind to B cells thereby promoting the selective expansion and survival of chicken B cells (Schneider *et al.*, 2004). In addition, it has been observed to selectively bind to duck B cells but not to other duck lymphocytes (Kothlow *et al.*, 2005; Fig. 21.1). Only recently duck BAFF has been cloned by RT-PCR and RACE (Guan *et al.*, 2007). The amino acid identities between biologically active duck and chicken BAFF and human BAFF is 97% and 78%, respectively. RT-PCR analysis showed that the duck BAFF gene is strongly expressed in the bursa of Fabricius. Purified recombinant duck BAFF is able to promote bursal cell survival.

TNF superfamily 15, the homologue of human TL1A (Kaiser *et al.*, 2005; Takimoto *et al.*, 2005), LPS-induced TNF-α factor (LITAF) (Malek *et al.*, 2004; Hong *et al.*, 2006) and CD154/CD40 ligand (Tregaskes *et al.*, 2005) have been cloned from chicken. Isolation of a cDNA encoding duck CD154/CD40L (duCD154) was achieved by RT-PCR involving RNA from duck splenocytes cultured in the presence of concanavalin A and LPS and primers based on the chicken CD154 nucleotide sequence (Fischer *et al.*, 2007; Table 21.1). Amino acid sequence identity to the previously identified chicken CD154 is 84% and to mammalian CD154 ~40%. When only the extracellular domain was expressed it was secreted, formed dimers and enhanced the proliferation of duck splenocytes. In mammals CD154 has been used as an adjuvant or in immunotargeting approaches to enhance vaccine responses. When ducks were immunized with constructs expressing the functional domain of duCD154 fused to the truncated DHBV core

protein they developed accelerated and enhanced core-specific antibody responses and showed enhanced proliferative responses of duck peripheral blood monocytic cells (PBMC) compared to ducks immunized with truncated core alone (Gares *et al.*, 2006). These results indicate that targeting of antigens to antigen-presenting cells (APC) with CD154 is an effective strategy to enhance immunological responses not only in mammals but also in birds.

Th2 Cytokines

The only avian species for which cytokines associated with Th2 responses have been identified is the chicken. Using a genomics approach based on conservation of synteny the chicken Th2 cytokines IL-3, IL-4, IL-5 and IL-13 have been isolated (Avery *et al.*, 2004). The Th2 response-promoting IL-10 has been isolated from an EST-library derived from RNA from caecal tonsils of *Eimeria*-infected chickens (Rothwell *et al.*, 2004). To our knowledge, nucleic acids encoding further cytokines of domesticated agricultural birds have not been identified to date.

CHEMOKINES

CXC Chemokines

The first chicken chemokine to be cloned was named 9E3/CEF4 (Bedard *et al.*, 1987; Sugano *et al.*, 1987). The protein encoded by this cDNA showed significant identity to both human IL-8/CXCL8 (51%) and human GRO-α (45%). As the exon:intron structure corresponds almost exactly to that of human IL-8/CXCL8 and the promoter contains potential regulatory sequences similar to those found in human IL-8/CXCL8, it was suggested that 9E3/CEF4 is the chicken orthologue of IL-8/CXCL8 (Kaiser *et al.*, 1999). 9E3/CEF4 is also known as cCAF (chicken chemotactic and angiogenic factor; Martins-Green and Feugate, 1998). However, more recently it was proposed that it would be called chicken CXCLi2 (chCXCLi2, "i" denoting inflammatory function) (Kaiser *et al.*, 2005).

Recent database entries provide sequence information on cDNA from duck and goose that show homology to chCXCLi2 cDNA (Table 21.1). Even the longest duck cDNA (291 nt; acc. no. AB205199, W.H. Chen and C. Xia, unpublished) does not contain a complete ORF but rather lacks part of the 5′ and 3′ coding region. It has the capacity to code for a protein that showed ~92% identity with chCXCLi2. The goose cDNA consists of 1157 nucleotides and contains a complete ORF (acc. no. AB213393, R.L. Guan and C. Xia, unpublished). It encodes for a protein of 103 amino acids. Amino acid sequence comparison of the entire protein revealed 92% identity with chicken chCXCLi2. When the partial duck amino acid sequence was compared with the corresponding region of the goose sequence 99% identity could be observed. Sequence identities of both the duck cDNA and goose cDNA with chicken K60/chCXCLi1, another well-known chicken CXCL chemokine, were ~70%. These homologies suggest that the sequences deposited as duck IL-8 and goose IL-8, respectively, may be the homologues of chCXCLi2.

CC Chemokines

Several genes of the chicken encoding CCL chemokines have been cloned and at least 15 have been identified in the chicken genome. Using subtractive hybridization technology Sreekumar *et al.* (2005a) identified three mRNAs in mitogen-stimulated splenic mononuclear cells of a duck with similarity to CCL chemokine sequences of chicken (Table 21.1). The predicted protein of one mRNA (AB163) consists of 90 amino acids, with a 22 amino acid N-terminal signal peptide. It showed 71% identity at the amino acid level with the described chCCL chemokine ah294 and about 52% identity with RANTES of human, bovine and guinea pig. The second mRNA (AB330) has been identified because of its high nucleotide identity to chicken MIP-1β

(81.6%). A cDNA thereof consisted of 715 nucleotides. The predicted ORF coded for a 90 amino acid protein that showed 76.6% identity to chicken MIP-1β and about 55–60% identity to known mammalian MIP-1β amino acid sequences. The third mRNA (AB187) has a size of 619 nucleotides and the coding capacity for a 91 amino acid protein. The coding region of AB187 did not reveal any close similarity with known chCCL chemokines. Amino acid sequence comparison revealed again the highest identity with chicken MIP-1β (36.2%). To see if this cDNA might show greater sequence homologies with recently cloned genes, we did a BLAST search and came up with the recently identified chCCLi1 to which AB187 showed ~41% identity. Basal level expression of all three molecules could be demonstrated in bursa of Fabricius, trachea, kidney and bone marrow. AB163 and AB187 could be detected in liver and lung, while the spleen showed the presence of AB163 and AB330. All three chemokine mRNAs are upregulated upon mitogen stimulation. As sequence information alone is not always sufficient to predict the biological activities of these molecules the true identity of the molecules preliminary designated MIP-1β and RANTES needs to be confirmed by appropriate biological assays.

In addition, two CC chemokines were identified in a duck spleen EST project, a CCL19 homologue (acc. no. DR766004) and CCL21 homologue (acc. no. DR764376) (Xia *et al.*, 2007). Like the chicken counterparts, these appear to be recognizably conserved across phylogeny. We have also identified the corresponding receptor CCR7 (C. Brusnyk, K. Ross and K. Magor, unpublished). The mammalian CCL19 and CCL21 chemokines are involved in trafficking of lymphocytes to the secondary lymphoid tissue, and further induce a proinflammatory cytokine profile in licenced dendritic cells (Marsland *et al.*, 2005). These chemokines may have potential as vaccine adjuvants.

Only for chickens, a C chemokine related to mammalian lymphotactin has been described (Rossi *et al.*, 1999). No homologues have been identified in other domesticated agricultural birds.

CELL SURFACE ANTIGENS

Anti-Chicken mAb Cross-Reacting with Turkey, Quail and Duck Leukocytes

Intensive studies of poultry immune systems depend on the availability of mAb against leukocyte surface antigens. No doubt, the most extensive studies of avian leukocyte phenotypic markers have been done using the chicken. From the panel of anti-chicken mAb which have been used to study the tissue distribution, ontogeny and function of lymphocyte populations in the chicken, most of these mAb react with turkey cells, some with quail cells but very few bind to cells of duck lymphoid organs thereby reducing the scope for investigations into cells of this species (Jeurissen and Janse, 1998; Table 21.2). Flow cytometric analysis of PBMC from individuals in five turkey lines revealed that certain anti-chicken CD8α mAb (3-298, 11-39) showed good cross-reactivity with the corresponding molecule on turkey cells. However, the mAb CT8 failed to detect the CD8α molecule in some turkeys, and there were large line differences in the ability to detect the epitope, suggesting, as is the case with chicken, polymorphism of the turkey molecule (Table 21.2). This suggestion is fostered by the observation that mAb 3-298 distinguishes two different populations in single-colour staining: $CD8\alpha^{bright}$ and $CD8\alpha^{dim}$ (Li *et al.*, 1999). In chicken, the $CD8\alpha^{dim}$ population of peripheral blood cells expressed CD8α as a homodimer in addition to CD4 (Luhtala *et al.*, 1997; Luhtala, 1998) whereas all $CD8\alpha^{bright}$ cells were CD4 negative and expressed CD8β as a CD8αβ heterodimer (Luhtala, 1998). Further experiments are needed to extend these observations to other avian species. In the same setting most of the anti-chicken CD4 mAb reacted with a determinant on turkey PBMC (Table 21.2). It should be pointed out that these mAb may not be recognizing the same molecule on turkey cells or that they occur on a different cell population in turkeys. However, certain anti-chicken mAb that were found to bind to epitopes on turkey PBMC and splenocytes recognized molecules that might represent the turkey homologues of chicken Bu-1 (chB6), CD4, CD8, CD28 and CD44 as these molecules show high sequence identities on the amino acid level to their chicken counterparts

TABLE 21.2 List of Monoclonal Anti-Chicken Antibodies Cross-Reacting with Turkey, Quail and Duck Cells

Antibody	Antigen	Specificity	Turkey	Quail	Duck	References
HIS-C7	CD45	All leukocytes	−	−	−	Jeurissen *et al.* (1988), Jeurissen and Janse (1998)
LT40	CD45	All leukocytes	nd	nd	(+)[a]	Paramithiotis *et al.* (1991), A. Bean, G. Reaiche, E. Bertram, J. Lowenthal and A. Jilbert, unpublished[b]
HIS-C1	Bu-1	B cells	+	−	−	Jeurissen *et al.* (1988), Jeurissen and Janse (1998)
AV20	Bu-1	B cells, subsets of monocytes/ macrophages	±	nd	−	Rothwell *et al.* (1996), Tregaskes *et al.* (1996), P. Kaiser, unpublished[c]; Southern Biotechnology, unpublished; S. Kothlow, B. Kaspers, U. Schultz, unpublished[d]; A. Bean, G. Reaiche, E. Bertram, J. Lowenthal and A. Jilbert, unpublished[b]
HIS-C12	IgM	IgM-positive B cells	+	+	−	Jeurissen *et al.* (1988), Jeurissen and Janse (1998)
M1	IgM	IgM-positive B cells	+	nd	nd	Chen *et al.* (1982), Van Nerom *et al.* (1997), Lawson *et al.* (2001)
CVI-ChIgG-47.3	IgG	IgG-positive B cells	+	+	−	Jeurissen *et al.* (1988), Jeurissen and Janse (1998)
AV-G3	IgG	IgG-positive B cells	+	nd	nd	Van Nerom *et al.* (1997)
CVI-ChIgA-46.5	IgA	IgA-positive B cells	+	−	−	Jeurissen *et al.* (1988), Jeurissen and Janse (1998)
CVI-ChIgL-47.5	Ig λ chain	All IgG-positive B cells	+	−	−	Koch and Jongenelen (1988), Jeurissen and Janse (1998)
CT1	Common thymocyte antigen	Thymocytes	nd	+	−	Chen *et al.* (1984), Coltey *et al.* (1989), A. Bean, G. Reaiche, E. Bertram, J. Lowenthal and A. Jilbert, unpublished[b]
CT3	CD3	T cells	−	−	−	Chen *et al.* (1984), Coltey *et al.* (1989), Char *et al.* (1990), Jeurissen and Janse (1998), A. Bean, G. Reaiche, E. Bertram, J. Lowenthal and A. Jilbert, unpublished[b]
AV29	CD4	Helper T cells	+	+	−	T.F. Davison, unpublished; Jeurissen and Janse (1998), Lawson *et al.* (2001)
CT4	CD4	Helper T cells	+	−	−	Chan *et al.* (1988), Coltey *et al.* (1989), Char *et al.* (1990), Li *et al.* (1999), P. Kaiser, unpublished[c]; Southern Biotechnology, unpublished; S. Kothlow, B. Kaspers, U. Schultz, unpublished[d]; A. Bean, G. Reaiche, E. Bertram, J. Lowenthal and A. Jilbert, unpublished[b]
2-35	CD4	Helper T cells	+	nd	nd	Luhtala *et al.* (1993), Li *et al.* (1999)
7-125	CD4	Helper T cells	+	nd	nd	Luhtala *et al.* (1993), Li *et al.* (1999)
2-6	CD4	Helper T cells	+	nd	nd	Luhtala *et al.* (1993), Li *et al.* (1999)
EP96	CD4	Helper T cells	nd	nd	−	A. Bean, G. Reaiche, E. Bertram, J. Lowenthal and A. Jilbert, unpublished[b]
CT8	CD8	Cytotoxic T cells	±	−	nd	Chan *et al.* (1988), Coltey *et al.* (1989), Char *et al.* (1990), Suresh *et al.* (1993), Rautenschlein and Neumann (1995), Li *et al.* (1999)
CVI-ChT-74.1	CD8α-chain	Cytotoxic T cells	−	−	−	Noteborn *et al.* (1991), Jeurissen and Janse (1998)

(*Continued*)

TABLE 21.2 (Continued)

Antibody	Antigen	Specificity	Turkey	Quail	Duck	References
11-39	CD8α-chain	Cytotoxic T cells	+	nd	nd	Luhtala *et al.* (1995), Li *et al.* (1999), P. Kaiser, unpublished[c]
3-298	CD8α-chain	Cytotoxic T cells	+	nd	−	Luhtala *et al.* (1997), Li *et al.* (1999), Lawson *et al.* (2001), Southern Biotechnology, unpublished; U. Schultz, unpublished[d]
EP42	CD8β-chain		+	nd	nd	Paramithiotis *et al.* (1991), Li *et al.* (1999)
AV-7	CD28	αβ T cells	+	nd	nd	Young *et al.* (1994), Lawson *et al.* (2001)
2-4	CD28	αβ T cells	nd	nd	+	Kothlow *et al.* (2005)
AV-6	CD44	Most cells including B and T cells, monocytes, subsets of epithelial cells	+	nd	(+)[a]	Lawson *et al.* (2001), Southern Biotechnology, unpublished; A. Bean, G. Reaiche, E. Bertram, J. Lowenthal and A. Jilbert, unpublished[b]
TCR1	γδ TCR	γδ T cells	−	−	−	Sowder *et al.* (1988), Coltey *et al.* (1989), Char *et al.* (1990), Jeurissen and Janse (1998), U. Schultz, unpublished[d]; A. Bean, G. Reaiche, E. Bertram, J. Lowenthal and A. Jilbert, unpublished[b]
TCR2	$\alpha\beta_1$ TCR	Subpopulation of αβ T cells	+	−	−	Chen *et al.* (1988), Cihak *et al.* (1988), Coltey *et al.* (1989), Char *et al.* (1990), Lawson *et al.* (2001), U. Schultz, unpublished[d], A. Bean, G. Reaiche, E. Bertram, J. Lowenthal and A. Jilbert, unpublished[b]
TCR3	$\alpha\beta_2$ TCR	Subpopulation of αβ T cells	−	−	−	Coltey *et al.* (1989), Char *et al.* (1990), U. Schultz, unpublished[d]; A. Bean, G. Reaiche, E. Bertram, J. Lowenthal and A. Jilbert, unpublished[b]
AV33	$\alpha\beta_{1/2}$ TCR	αβ T cells	−	+	−	T.F. Davison, unpublished; Jeurissen and Janse (1998)
CVI-ChNL-68.1		Mononuclear phagocytes	+	+	+	Jeurissen *et al.* (1988), Jeurissen and Janse (1998)
CVI-ChNL-74.2		Subpopulation of tissue macrophages	−	−	−	Jeurissen *et al.* (1992), Jeurissen and Janse (1998)
K1		Monocytes/ macrophages, thrombocytes	+	nd	+	Kaspers *et al.* (1993), Lawson *et al.* (2001), Kothlow *et al.* (2005)
CVI-ChNL-68.3	MHCII	APC, B cells	−	−	−	Jeurissen *et al.* (1989), Jeurissen and Janse (1998)
2G11	MHCII	APC, B cells	+	nd	nd	Guillemot *et al.* (1986), Lawson *et al.* (2001)
F21-2	MHCI	Most nucleated cells	+	nd	+	Salomonsen *et al.* (1987), P. Kaiser, unpublished[c]; Southern Biotechnology, unpublished; U. Schultz, unpublished[d]
F21-21	β2 m	Most nucleated cells	+	nd	−	Skjodt *et al.* (1986), Lawson *et al.* (2001), Southern Biotechnology, unpublished; A. Bean, G. Reaiche, E. Bertram, J. Lowenthal and A. Jilbert, unpublished[b]

nd: not determined.
[a]Flow cytometric analysis of Pekin duck PBMC, ⩽7% positive cells.
[b]Flow cytometric analysis of Pekin duck PBMC.
[c]Flow cytometric analysis of turkey PBMC.
[d]Flow cytometric analysis of Pekin duck thymocytes and splenocytes.

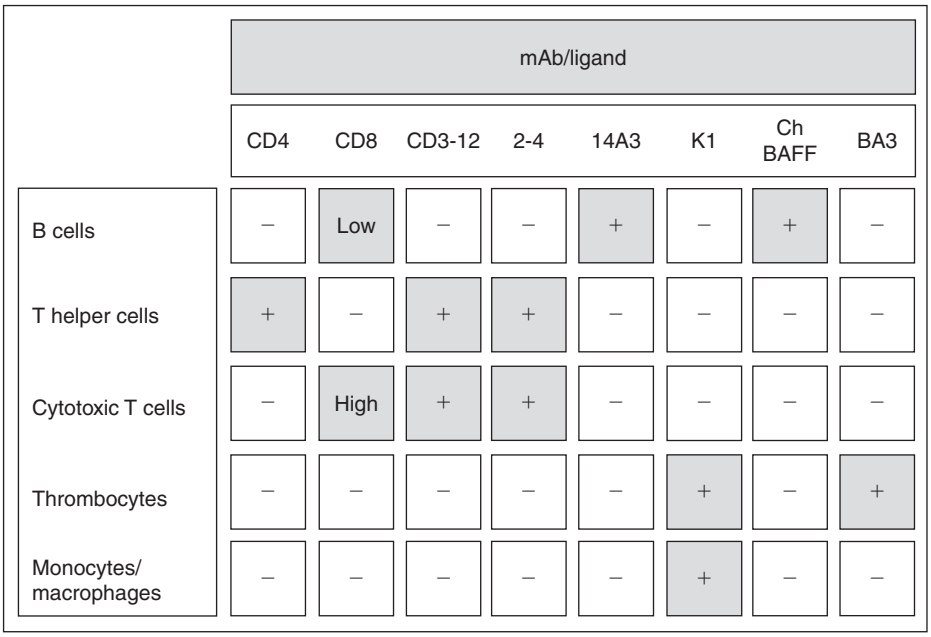

FIGURE 21.1 Range of mAb suitable for phenotyping the major leukocyte populations in ducks. mAb specificity: CD4 (CD4 antigen), CD8 (α-chain of CD8), 3-12 (conserved peptide of the ε-chain of CD3), 2-4 (CD28), 14A3 (duck Ig light chain), K1 (common antigen on thrombocytes and monocytes/macrophages), chBAFF (BAFF receptor on B cells), BA3 (antigen on thrombocytes; Bertram *et al.*, 1998).

(84–95%; Lawson *et al.*, 2001). In addition, immunoprecipitation and western blotting showed that mAb 3-298 precipitated a 33–35-kDa polypeptide similar to the relative molecular mass of the chicken CD8 molecule (Li *et al.*, 1999). With the mAb TCR2 (which recognizes TCRαβ), M1 (IgM), K1 (an antigen on monocyte/macrophages and thrombocytes), F21-21 (β_2 microglobulin), and 2G11 (major histocompatibility complex (MHC) class II antigen) five additional mAbs have been made that bind to epitopes on turkey PBMC and splenocytes (Lawson *et al.*, 2001; Table 21.2). With respect to the cross-reactivity of mAb AV20, which recognizes chB6, conflicting observations have been reported. This might be explained by the differences in the genetic backgrounds of the birds resulting in minor modifications to the epitope (P. Kaiser, unpublished; Southern Biotechnology, unpublished data; see Table 21.1).

The fact that certain mAb specific for chicken T cell markers: CT3 (recognizing CD3), CT4 (CD4), CT8 (CD8α), TCR1 (TCRγδ), TCR2 (TCRαβ_1) and TCR3 (TCRαβ_2) do not cross-react with quail cells has been used to confirm the identity of chicken cells in chicken–quail chimaeras (Coltey *et al.*, 1989; Char *et al.*, 1990; Table 21.1). However, an mAb detecting a common chicken thymocyte antigen did cross-react with quail thymocytes (Coltey *et al.*, 1989). Interestingly, an mAb which does not discriminate between TCR2(αβ_1) and TCR3(αβ_2) does show cross-reactivity with quail cells. One possible explanation is that this mAb recognizes a less polymorphic epitope. In addition, the anti-chicken CD4, mAb AV29, reacts with a subset of quail lymphocytes. Furthermore, mAb have been identified that bind to IgM^+ and IgY^+ B cells and also mononuclear phagocytes, respectively (Jeurissen and Janse, 1998).

Unfortunately, of the group of anti-chicken antibodies specific for B and T cell determin-ants, none cross-reacted with duck cells (Jeurissen and Janse, 1998; S. Kothlow, B. Kaspers and U. Schultz, unpublished; A. Bean, G. Reaiche, E. Bertram, J. Lowenthal and A. Jilbert, unpublished; Table 21.2). It was only after mAbs specific for duck CD4 and CD8, respectively, had been generated that the mAb which recognized chicken CD28 was shown to react with the vast majority of $CD4^+$ and a subset of $CD8^+$ splenocytes (Kothlow *et al.*, 2005; Table 21.3; Fig. 21.1). However, two mAb were found that allowed the identification of duck mononuclear phagocytes (Jeurissen and Janse, 1998; Kothlow *et al.*, 2005). One of them (K1) detects a common

TABLE 21.3 List of Cloned Duck Cell Surface Molecules[a]

Molecule	Accession Number	References
CD3ε-chain	AF378704; AY738731, AY738734[b]	S.W.S. Chan, D.L. Middleton, M. Lundqvist, G.W. Warr and D.A. Higgins, unpublished; Kothlow *et al.* (2005)
CD4	AF378701; AY738732, AY738736[b]	S.W.S. Chan, D.L. Middleton, M. Lundqvist, G.W. Warr and D.A. Higgins, unpublished; Kothlow *et al.* (2005)
CD8α-chain	AF378373; AY738733, AY738735[b]	S.W.S. Chan, D.L. Middleton, M. Lundqvist, G.W. Warr and D.A. Higgins, unpublished; Kothlow *et al.* (2005)
CD25	DQ299949	J.Y. Wang, J.Y. Zhou and J. Qi, unpublished
CD44 isoform a	AY029553	S.W.S. Chan, D.L. Middleton, G.W. Warr and D.A. Higgins, unpublished
CD44 isoform b	AF332869	S.W.S. Chan, G.W. Warr, D.L. Middleton and D.A. Higgins, unpublished
CD44 isoform c	AY032667	S.W.S. Chan, D.L. Middleton, M. Lundqvist, G.W. Warr and D.A. Higgins, unpublished
CD58	AY032731	S.W.S. Chan, D.L. Middleton, M. Lundqvist, G.W. Warr and D.A. Higgins, unpublished
MHC class I	AY885227; AY294416	Moon *et al.* (2005) and Mesa *et al.* (2004)
TCRα-chain	AF323922[c], AF542183[c]	S.W.S. Chan, G.W. Warr, D.L. Middleton, M.L. Lundquist and D.A. Higgins, unpublished; S.W.S. Chan and D.A. Higgins, unpublished
TCRβ-chain	AF068228[c]; AY039002	Chan *et al.* (1999), S.W.S. Chan and D.A. Higgins, unpublished
TCRγ-chain	AF378702; AY294416	S.W.S. Chan, O.K.H. Ko and D.A. Higgins, unpublished
TCRδ-chain	AF415216	S.W.S. Chan and D.A. Higgins, unpublished

[a]Pekin duck (*Anas platyrhynchos*) except of sequences indicated.
[b]Muscovy duck (*Cairina moschata*).
[c]Partial coding sequence.

antigen on chicken monocytes/macrophages and thrombocytes, and might therefore prove to be a valuable tool for depleting PBMC preparations of myeloid cells (Fig. 21.1). In addition, it has been shown that it is possible to detect duck Ig with mammalian antisera raised against chicken Ig (Halpern *et al.*, 1987).

Evidence for T and B Cell Populations in Ducks

Until recently, studies of the immune system of ducks have not been successful in classifying lymphocytes phenotypically as cells resembling mammalian/chicken T and B lymphocytes (Halpern *et al.*, 1987; Jeurissen and Janse, 1998; Miller, D.S. *et al.*, 2004; Table 21.2). However, duck lymphocytes have been shown to have some of the functional characteristics of T cells as they respond to a range of mitogens, with PHA being the best studied (Higgins and Teoh, 1988). Resembling the requirements for activation of mammalian lymphocytes, the mitogenic response of duck PBMC is dependent on adherent cells (Higgins, 1992). In addition, a polyclonal rabbit antiserum specific for a conserved cytoplasmic domain in the CD3ε molecule has been reported to bind to a population of duck lymphocytes with a distribution expected of T cells (Bertram *et al.*, 1996). This anti-human CD3 rabbit antiserum immunoprecipitated a 23 kDa protein from duck lymphoblast lysates. The size is consistent with the molecular mass described for human CDε. Furthermore, B cells have been identified in duck lymphatic tissues using goat

anti-duck Ig antisera (Ellsworth and Ellsworth, 1981). Goat antibodies hold the advantage that they were not bound via Fc receptors, thus occurrence of non-specific binding can be excluded.

Antigens Expressed on Duck Lymphocyte Subsets

The CD4 and CD8 Antigen

CD4 is a single-chain transmembrane glycoprotein expressed on most thymocytes and on a subset of T cells (Parnes, 1989). In addition, in humans CD4 is also expressed on macrophages and granulocytes. CD4 acts as a cellular adhesion molecule binding class II molecules. It stabilizes the interaction of MHC class II restricted T cells (Doyle and Strominger, 1987). Interactions of CD4 with MHC class II molecules are crucial during thymic development and subsequently for the function of single-positive CD4 T cells. CD8 occurs as a homodimer of two CD8α-chains or as a heterodimer consisting of a CD8α- and CD8β-chain. CD8 is required for the development of cytotoxic T cells and functions as an adhesion molecule which binds to MHC class I molecules. In addition, in the chicken the CD8 molecule is also found on some splenic and gut natural killer (NK) cells which both express the CD8$\alpha\alpha$ homodimer (Göbel *et al.*, 1994). The genes coding for chicken CD4 and CD8 were the first CD antigens identified in an avian species (Tregaskes *et al.*, 1995; Koskinen *et al.*, 1999). cDNA for Pekin and Muscovy duck CD4 and the α-chain of CD8 have been amplified by RT-PCR taking advantage of sequence information recently deposited by Higgins and coworkers (acc. no. AF378701, AF378373, S.W.S. Chan, D.L. Middleton, M. Lundqvist, G.W. Warr and D.A. Higgins, unpublished; Table 21.3). Overall, the amino acid sequence of the Pekin duck CD4 precursor showed a higher degree of identity to avian (Muscovy duck 91.3%, chicken 60.2%) than to mammalian sequences (human 19.2%). Similar levels of identity were observed for Pekin duck CD8: Muscovy duck 86.5%, chicken 59.6% and human 25.1%. Duck CD4- and CD8-transfected 293T cells proved to be suitable to generate mAbs reacting with subsets of duck splenocytes, thymocytes and PBL preparations (designated duCD4-1, duCD4-2, duCD8-1, duCD8-2; Kothlow *et al.*, 2005; Fig. 21.1). Earlier attempts using duck thymocytes or mitogen-stimulated duck PBMC for immunization had produced numerous mAb which reacted with duck lymphocytes however, it had not been possible to align the reactivities to functionally defined subpopulations (Higgins, 1996; Jilbert and Kotlarski, 2000). Using two-colour immunofluorescence staining, duck thymocyte preparations showed the presence of double-negative, double-positive and single-positive CD4 or CD8 cell populations, which represent the typical maturation stages during thymic T cell development. In contrast, peripheral lymphocytes were either $CD4^+$ or $CD8^+$. Nonetheless, a very small percentage of cells were shown to be double positive. While $CD4^+$ cells are represented by a homogenous population, $CD8^+$ cells were recognized as two populations with different staining patterns: $CD8^{high}$ and $CD8^{low}$. This resembles data obtained with young chicks, where $CD8^{bright}$ cells express the CD8$\alpha\beta$ heterodimer while $CD8^{dim}$ express the CD8$\alpha\alpha$ homodimer. In older chickens this phenotype is lost and all $CD8^+$ cells express the heterodimer (Tregaskes *et al.*, 1995). The analogous situation does not apply to duck lymphocytes, as shown by the unexpected observation that the vast majority of duck bursal cells react with duCD8-1 and duCD8-2. Confirming the flow cytometric data, CD8α gene expression can be demonstrated in the bursa, while CD4 and CD3ε transcripts are not detected (Kothlow *et al.*, 2005). In addition, more than 90% of bursal cells were double positive using two-colour immunofluorescence staining involving duCD8-1 in combination with 14A3, a recently generated duck Ig light chain specific mAb (see below and Fig. 21.1). Furthermore, three distinct cell populations could be identified in spleen cell preparations under these conditions: double-negative cells representing $CD4^+$ lymphocytes, $CD8^{high}/14A3^-$ cells which could be cytotoxic T lymphocytes and, finally, $CD8^{low}/14A3^+$ cells representing B lymphocytes. By CD3ε staining only, $CD8^{high}$ cells can be identified as T cells while the $CD8^{low}$ cells were $CD3^-$ but express surface Ig. Therefore, it should be emphasized that the $CD8^{low}$ cell population in ducks is not identical to the $CD8^{dim}$ cell population that has been described in young chickens (Tregaskes *et al.*, 1995). To our knowledge CD8 antigen expression

on B cells has not been reported in other species. It is tempting to speculate about the role of CD8 expression in duck B cell development and B cell function. Preliminary results, at least, suggest that the CD8 molecule is functional as tyrosine phosphorylation can be observed upon binding of duCD8-1 to bursal cells (U. Schultz, unpublished). It is further intriguing to know whether this phenomenon is restricted to ducks and maybe other anseriforms or can be found outside this order. However, it should be noted that the newly developed mAb for CD4 and CD8 failed to stain lymphocytes from Muscovy ducks suggesting that the epitopes recognized are not conserved among these two species.

The CD3/TCR Complex

As well as in mammals, the chicken CD3 complex (γ, δ, ε, and ζ chains) is found on the surface of T cells in association with the T cell receptor (TCR) complex (either αβ or γδ) and together are required for antigen recognition (Chen *et al.*, 1986). mAb reacting with CD3 permit the identification of all peripheral T cells, regardless of their TCR and are therefore used to distinguish T from B cells. In addition, some chicken lymphoid cells express cytoplasmic CD3 molecules but not TCR molecules. These cells, which have been identified in the gut and in embryonic spleen, are considered to be avian NK cells (Göbel *et al.*, 1994). Based on publicly available sequence information for Pekin duck CD3ε (acc. no. AF378704, S.W.S. Chan, D.L. Middleton, M. Lundqvist, G.W. Warr and D.A. Higgins, unpublished data; Table 21.3) cDNA clones comprising the entire ORF of Pekin and Muscovy duck CD3ε were obtained (Kothlow *et al.*, 2005; Table 21.3). The precursor proteins of Pekin and Muscovy duck CD3ε reveal 86.5% amino acid identity which decreased to 61.7% and 39.3%, respectively, when compared to chicken and human CD3ε polypeptides. CD3ε gene expression was observed in PBMC, spleen and thymus, whereas transcripts were not found in bursa of Fabricius and liver. Duck CD3ε can be detected by intracellular staining using mAb CD3-12 which recognizes the highly conserved epitope PPVPNPDYEP of CD3ε, which is also present in the duck molecule. Double staining with mAb CD3-12 and mAb2-4 revealed that the majority of $CD3^+$ cells are 2-4 positive confirming that the chicken CD28-specific antibody is also a valuable pan-T cell marker in ducks (Kothlow *et al.*, 2005; Fig. 21.1). T cell subdivision is not well studied in other birds than chicken because none of the chicken-specific TCR mAbs cross-reacted with the respective molecules on duck cells (Table 21.3). It is encouraging, therefore, that sequence information on the entire coding sequence of TCRβ, γ, and δ has become available recently (acc. no. AF068228, AY039002, Chan *et al.*, 1999; S.W.S. Chan and D.A. Higgins, unpublished data; acc. no. AF378702, S.W.S. Chan, O.K.H. Ko and D.A. Higgins, unpublished data; acc. no. AF415216, S.W.S. Chan and D.A. Higgins, unpublished data). For TCRα at least partial sequences have been obtained (acc. no. AF323922, S.W.S. Chan, G.W. Warr, D.L. Middleton, M.L. Lundqvist and D.A. Higgins, unpublished data; acc. no. AF542183, S.W.S. Chan and D.A. Higgins, unpublished data; Table 21.3). Hopefully this will lead to the production of mAb which allow the identification and study of T cell subsets in this species.

The CD28 Antigen

The chicken CD28 is a glycoprotein which, in contrast to mammalian CD28, does not form disulphide linked homodimers due to the lack of cysteine residues (Young *et al.*, 1994). Its presence has been demonstrated on all peripheral T cells carrying the TCRαβ and on subpopulations of TCRγδ-bearing T cells. mAb2-4, which was shown to react with the chicken CD28 molecule, was found to cross-react with subsets of duck PBMC. Two-colour staining of duck splenocytes revealed that virtually all CD4 cells are mAb2-4^+, in addition to the vast majority of $CD8^{high}$ cells; while the $CD8^{low}$ population was found to be mAb2-4^-. It is worth noting that when using mAb2-4 in combination with recombinant fluorescent-labelled chBAFF, mutually exclusive subsets of cells are stained. In conclusion, when used in combination with a CD8-specific mAb the mAb2-4 is able to discriminate between $CD8^{high}$ cytotoxic T cells and $CD8^{low}$ B cells (Kothlow *et al.*, 2005; Fig. 21.1).

SURFACE IMMUNOGLOBULIN

Surface membrane-bound Ig is a characteristic feature for B cells in most species. Antibodies raised to duck Ig, however, seemed to bind not only B cells but also react with a large proportion of duck lymphocytes from blood, spleen, thymus and bursa of Fabricius as well as erythrocytes impeding the identification of B cells (Higgins and Chung, 1986). However, we have recently developed a mAb to the duck Ig light chain (14A3) that reacted with the IgY molecule of both Pekin and Muscovy ducks, and also with goose, though not with chicken IgY. In addition, western blot analysis revealed that 14A3 reacted with IgA purified from Pekin duck bile (Kothlow *et al.*, 2005; Fig. 21.1). mAb 14A3 stained >90% of bursal cells but only minor populations in spleen and blood cell preparations. Two-colour immunofluorescence analysis with mAb and fluorescent-labelled chBAFF revealed a distinct double-positive cell population in spleen and PBMC with only few single-positive cells. The lack of double-positive cells in the thymus reflects the virtual absence of B cells in this organ. Taken together, these studies confirm that mAb 14A3 is a valuable tool to identify B cells in ducks and geese (Fig. 21.1).

MAJOR HISTOCOMPATIBILITY COMPLEX

Comparison of vertebrate MHC genomic regions shows it is the most dynamic part of the genome (Kelley *et al.*, 2005). Polygeny and polymorphism contribute to the breadth of the immune response. The number of functional and defunct MHC loci can differ greatly in each species, and no orthologous genes can be identified between orders. The MHC of the chicken has been referred to as the "minimal MHC" (Kaufman, 2000; see Chapter 8). There are two clusters of MHC genes, the B locus and the Rfp-Y locus, that are located on the same microchromosome (Miller *et al.*, 1996). The B locus contributes all the hallmarks of the MHC. It contains 19 genes within 92 kb and therefore is very compact (discussed in Chapter 8). Within the B locus, there are two class I genes that flank either side of the transporters for antigen processing (TAP) genes. Recent nomenclature refers to these as the major (BF2) and minor (BF1) MHC class I loci (Miller, M.M. *et al.*, 2004). Kaufman argues that in this organization the MHC class I and transporter proteins have an opportunity to co-evolve to function together, and evolutionary forces select for inactivation of the redundant loci (Kaufman, 2000). The limitation to one major MHC class I locus will have significant implications for the immune responses of the chicken. This is clearly demonstrated in the response of chickens to the Rous sarcoma virus (RSV), with the MHC genotype conferring either protection or none (Wallny *et al.*, 2006).

The MHC of other birds appears to have undergone extensive duplications and the minimal MHC of the domestic chicken is not the norm for all birds. The red jungle fowl has 14 copies of MHC class I like genes, 3 of which are pseudogenes, suggesting the minimal MHC of the chicken is a derived characteristic (International Chicken Genome Sequencing Consortium, 2004). The MHC of the quail has been completely sequenced and has the same overall organization as that of chicken. However, it has undergone duplication in several regions. There are 7 MHC class I, 10 MHC class IIβ, 4 NK receptors, 6 lectin and 8 B-G genes. MHC class I genes flank the TAP loci; however, there are 4 MHC class I genes and 3 pseudogenes. Of these, one gene seems most likely to be the orthologue of the chicken BF2 gene, and two loci are weakly expressed. The duck MHC class I region encodes five class I genes that lie adjacent to the TAP2 gene (Moon *et al.*, 2005). No evidence for class I genes on the other side of the TAP genes was evident, nor was there evidence of other class I genes in the genome by Southern blotting. Using a northern blotting approach and allele specific oligonucleotide probes, we showed that the duck predominantly expresses only one gene, that adjacent to the TAP2 gene, Anpl UAA (Mesa *et al.*, 2004). Consistent with expression of one gene, we were able to amplify only one or two allelic sequences by reverse transcription PCR from wild mallards (C. Mesa, J. Parks-Dely, D. Moon, J. Wong and K. Magor, unpublished). The predominant expression of a single MHC class I locus is

expected to have significant functional consequences, influencing the selection of the TCR repertoire and NK cell receptor repertoire, as well as the peptide repertoire that can be presented. The cytotoxic cell responses of ducks are likely to be easily circumvented by viruses through mutation, which may play a role in the ease with which influenza exploits the duck as a host.

SECRETED ANTIBODIES

Birds have three classes of immunoglobulins: IgM, IgA and IgY. IgM and IgY are present in serum, while IgA is expressed in a variety of secretions (see Chapter 6). The IgY is an avian version of IgG, which is sufficiently different from IgG to warrant that name (Warr *et al.*, 1995). In addition, ducks and geese have a smaller version of IgY, called IgYΔFc, a secreted antibody composed of two C region domains.

Descriptions of avian antibodies in birds other than ducks are limited. A characterization of serum antibodies of turkeys showed cross-reactivity with commercially available mAb; the mAb AV-G3 detects turkey IgG, while the mAb M1 reacts with IgM (Van Nerom *et al.*, 1997). A characterization of serum antibodies of the ostrich showed they were not cross-reactive with antibodies against Ig of many species including chicken (Cadman *et al.*, 1994). Early analyses of Ig of quail and pheasants were carried out (Leslie and Benedict, 1969; Ch'ng and Benedict, 1981). The cross-reactivity of many mAb against chicken antigens was tested in ducks, turkeys and quails; none reacted with duck Ig, two were found to react with quail antibodies and several were found to react with antibodies of turkeys (Jeurissen and Janse, 1998; Table 21.1).

Duck Ig genes are the best characterized, and the Ig heavy chain locus has been sequenced on overlapping clones from a D gene segment through the upsilon gene (Magor *et al.*, 1999; Lundqvist *et al.*, 2001). There are three clusters of heavy chain gene exons but their organization is unusual. The mu (μ) gene is followed by an inverted alpha (α) gene, and upsilon (υ) lies furthest downstream in the locus. This organization will make splicing for Ig class switching cumbersome. Switching to υ involves normal excision of the switch circle, but switching to α requires the inversion of this fragment in the locus. Switching to α is delayed in ontogeny, and transcripts are barely detectable until ducks are about two weeks old. Ducklings are likely to encounter influenza A before IgA can play a role in the defence. The υ gene encodes three different forms of IgY heavy chain through alternate splicing (Magor *et al.*, 1994). Typically the ratio of full length and truncated IgY varies between individuals, but the truncated IgY predominates later in an immune response. After repeated injections of antigen, IgYΔFc made up 85% of the specific antibody to bovine serum albumin (Grey, 1967) or *E. coli* (Humphrey *et al.*, 2004). Since this antibody lacks the Fc portion, it cannot participate in secondary effector functions, such as complement fixation, opsonization and Fc mediated macrophage clearance of viruses. Presumably it functions primarily in neutralization.

CELL LINES

Numerous lines of chicken B and T cells and various lines of non-lymphoid cells have been established using Marek's disease virus, RSV and other leukosis viruses, and reticuloendotheliosis virus (REV). Depending on the age of the bird, the origin of cells, e.g. bone marrow cells versus splenocytes, the activation status and the strain of virus employed, infection resulted in transformed populations containing predominantly T cells, B cells or monocytes/macrophages (Witter *et al.*, 1986; Marmor *et al.*, 1993). REV which is the most versatile transforming virus is not only pathogenic in chicken and turkey but also in ducks (Li *et al.*, 1983) and geese (Drén *et al.*, 1988). Higgins and coworkers reported the establishment of lymphoblastoid cell lines from organs of ducks infected with REV-T, a replication-defective strain (Chan *et al.*, 1999). In uncloned lines the presence of T and B cells was suggested by the expression of the β-chain of the TCR and the expression of Ig polypeptides. However, the cloned lines that were obtained

appeared to be of the αβ T cell lineage. The failure to generate B cell lines was explained by stringent growth requirements for B cells, e.g. cytokines derived from T cells (Chan *et al.*, 1999).

Kaiser and coworkers succeeded in establishing a turkey macrophage cell line (LSTC-IAH30) after challenge of adherent PBMC with acutely transforming avian leukosis virus (ALV) (Lawson *et al.*, 2001). By flow cytometric analysis it was shown that LSTC-IAH30 cells are positive for markers recognized by M, K1 (detects a common antigen on chicken thrombocytes and macrophages), F21-21 (chicken β2 microglobulin) and possibly M1 (detects chicken IgM; Table 21.2) but not KUL01 (chicken macrophage marker; Mast *et al.*, 1998). Furthermore production of nitric oxide has been demonstrated for LSTC-IAH30 cells and they have, therefore, been used to measure turkey IFN-γ activity as a function of nitrite accumulation (Lawson *et al.*, 2001).

Furthermore, HD-11 cells, a well-known chicken macrophage-like cell line, proved to be suitable to measure duck IFN-γ activity, however, duck IFN-γ had a specific activity that was about 16-fold lower than chicken IFN-γ that served as control (Huang *et al.*, 2001).

CEC-32 cells were reported to have been established from chicken embryo fibroblasts (Kaaden *et al.*, 1982). Due to their virus susceptibility and sensitivity to IFN, they have been successfully used to study the functions of the chicken and duck IFN systems. A more recent comparison of the karyotype of CEC-32 cells with chicken and quail karyotypes revealed that the CEC-32 cell line might not have originated from chicken but have come from quail (Zoller *et al.*, 2000).

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22 ECOIMMUNOLOGY

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INTRODUCTION
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INTRODUCTION

One of the central tenets of evolutionary biology is that the favourable heritable traits increase in abundance due to natural selection. Infectious diseases often interfere with reproductive success and, as a consequence, evolutionary pressure will favour individuals that are effective at resisting attacks from parasites and pathogens. Although all species have evolved defence systems, individuals vary greatly in their resistance to disease. Ecoimmunology seeks to address why we see variation in immune responses among individuals and across species. An ecoimmunological approach takes an explicitly evolutionary perspective by hypothesizing that immunity is costly (e.g. energetically expensive) and thus subject to tradeoffs. Under certain conditions animals may have better reproductive success if they invest less in immunity and more in reproduction (Viney *et al.*, 2005). Conversely, if pathogen pressure is high, animals may need to increase investment in immunity at the expense of reproduction.

Examining immunology in an ecological and evolutionary perspective places immune activity within the framework of how organisms interact with their environment. For example, the ecology and evolution of immune function and disease is influenced by population-level phenomenon and social environment. In addition, in many clinical and laboratory studies variation among individuals is considered statistical noise. However, an ecoimmunological approach is interested in explicitly determining the causes and consequences of individual variation. This is because differential survival and reproduction among individuals is a key mechanism of evolutionary change (Darwin, 1859) and variation is assumed to represent, in part, the outcome of natural selection, as well as the variation upon which selection can act in a population. Generally, ecoimmunology addresses biological questions related to life history theory, a

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sub-field of evolutionary biology, which examines the strategies that have evolved over time to govern investment in reproduction (Ricklefs and Wikelski, 2002). Because maintenance of the immune system requires resources, optimal strategies of immune investment have evolved in the context of tradeoffs and constraints (Tella *et al.*, 2002; Schmid-Hempel, 2003). For example, maintaining a functional immune system requires energy and scarce nutrients (Klasing, 1998). Therefore, resources invested in immune system self-maintenance are not available for investment in reproduction. In addition, responding inappropriately (i.e. too strongly or too weakly) to perceived threats can have a strong influence on reproduction (Raberg *et al.*, 1998; Graham, 2002).

In contrast to domesticated birds such as chickens, and to a lesser degree turkeys and ducks, there is a paucity of information on immune responses in free-living species. Most often studies on immune responses are performed using birds with unknown histories and, with few exceptions, long-term studies are problematic. As a consequence, information on immune responses in a given species is often incomplete. Moreover, major differences such as whether a species is altricial (developmentally immature at hatching lacking feathers, eyesight and remaining in the nest for a period of time) versus precocial (developmentally mature at hatching with feathers, eyesight and the ability to leave the nest shortly after hatching) has a profound impact on immunological needs. The application of immunology to test life history theory in free-living bird species is an emerging field and few systematic studies have been conducted. Accordingly, studies tend to be fragmented with no clear model species. For this reason, interpretations of many studies should be considered preliminary as fundamental assumptions in the field are tested and reevaluated. In this chapter, we will discuss the problems associated with ecoimmunological studies, evaluate differences and similarities in immune responses among free-living avian species and, where appropriate, make comparisons with domestic species. We will also discuss how immunological responsiveness may reflect on ecological factors, differential selection pressures and evolutionary outcomes.

ASSAYS TO ASSESS IMMUNE FUNCTION IN FREE-LIVING BIRDS

The selection of the proper techniques presents major problems for the study of immune responses in free-living avian species. For many precocial and altricial species single-point measures are the only approach that can be used. If birds can be followed over time by recapturing parents using nets, or at nest (box) sites or by using nestlings in accessible nests, multiple-point assays can be conducted. The use of antigens to experimentally evaluate humoral and cell-mediated immune (CMI) responses in free-living birds is limited to antigens unrelated to pathogens (e.g. sheep red blood cells (SRBC), bovine serum albumin (BSA), keyhole limpet haemocyanin (KLH)), inactivated pathogen-derived antigens (e.g. inactivated Newcastle disease virus (NDV)) and lectins (e.g. phytohaemagglutinin (PHA) and concanavalin A (ConA)). If birds can be kept in captivity it may be possible to use pathogens, but the use of free-living birds in captivity may cause problems related to stress or nutritional imbalances causing aberrant immune responses.

Single-Time Point Measures

Single-time point assessments are generally used for monitoring the health of an individual at the time of assessment. The most common techniques are counts of leukocytes or ratios of heterophils to lymphocytes (reviewed in Norris and Evans, 2000). The effectiveness of single assessments is limited, however, because it is difficult to determine whether immune measures at a particular time point reflect immunocompetence or response to a recent infection.

The relative weight or volume of lymphoid organs such as the spleen or in juvenile birds, the bursa of Fabricius and thymus are often used if birds can be euthanized at the time of capture (Møller and Erritzoe, 1998; Ardia, 2005a). These measures are also sensitive to individual variation, overall health status or past activity and enlarged spleens may reflect pathological processes

and/or high immunocompetence. A recent study by Ardia (2005a) elucidates some of these problems. Nestling European starlings (*Sturnus vulgaris*) were inoculated with saline or PHA by subcutaneous injection in the wing web. Spleen sizes were highly variable among individuals after inoculation with saline indicating that the use of spleen size in comparison within and among a species is likely to be confounded by high variability. PHA stimulation did not cause enlargement of spleens relative to saline-injected controls (Ardia, 2005a). Assessing the bursa of Fabricius or thymus is relatively uncommon in field studies due to their small sizes. Moreover, in most species the bursa of Fabricius and the thymus involutes during sexual maturity (Kendall, 1980).

A promising single-time point measure is the examination of levels of constitutive innate or natural antibodies (NAb). These antibodies, which are mostly IgM but also include IgY (the avian homologue of IgG) and IgA, are most likely the result of responses to cross-reactive epitopes present in the natural environment of the host (e.g. intestinal tract) and do not require experimental antigen exposure. NAb react to a diversity of epitopes making them useful to assess levels of immunocompetence among individuals (Matson *et al.*, 2005). This technique is highly repeatable and is effective in a wide range of avian species (Matson *et al.*, 2005). The levels of NAb may reflect differences in genetic potential for acquired immune responses based on studies in chickens. Levels of NAb to rabbit red blood cells increased in chickens selected for acquired responses to SRBC but only after 14 generations (Cotter *et al.*, 2005) and higher NAb titres to other antigens were associated with selection for increased acquired responses to SRBC (Parmentier *et al.*, 2004). However, body condition needs to be carefully monitored in free-living birds because this may influence NAb titres. In nestling Leach's storm petrels (*Oceanodroma leucorhoa*: Procellariformes) NAb titres increased during nestling development but were inversely proportional to wing growth, suggesting an energetic tradeoff in developing nestlings (Mauck *et al.*, 2005).

NAb often react with SRBC or other red blood cells and can confound results when birds are experimentally immunized with SRBC. Testing of sera for antibodies at the time of first immunization is essential, but not always included in the experimental designs, and will reduce or eliminate the potential confounding factor of NAb. Another excellent single-time point assay uses enzyme-linked immunosorbent assays (ELISA) to determine general serum immunoglobulin levels, as well as specific antibodies, in free-living birds (Martinez *et al.*, 2003), but species-specific antisera may need to be generated for these assays (Smits and Baos, 2005). In addition, for those species from which it is possible to collect larger quantities of blood ($>500\,\mu l$), it is possible to use a variety of standard laboratory techniques, such as bactericidal competence and acute phase protein concentration, since many of these measures appear to be correlated on an individual level (Matson *et al.*, 2006).

Multiple-Time Point Assays

Multiple-time point assays are used to examine immune responses to novel antigens or mitogens. These assays allow the use of standardized experimental designs and measurements in which responses to the inert challenges are assumed to predict levels of immune activity to pathogens. Most assays stimulate activity in the two arms of the vertebrate acquired immune system: cell-mediated and humoral immune responses. Under field conditions, CMI responses are almost universally assessed through inoculation of PHA into the wing web, although in some species like penguins the foot web is used. PHA stimulates T-cell proliferation and secretion of cytokines that recruit granulocytes. Swelling is due to the infiltration of circulating granulocytes and oedema; thus, swelling reflects a T-cell-mediated inflammatory response and not necessarily T-cell proliferation. The response was historically assessed in comparison to change in thickness relative to a control injection. However, a recent meta-analysis concluded that the control injection was unnecessary (Smits *et al.*, 1999). The PHA technique has the advantage of not requiring laboratory work and therefore is commonly used in field studies. However, because the actual measurements are somewhat subjective, the test is prone to inter-observer variation (though levels of intra-observer variation can be quite low), thus greatly limiting the

comparison of PHA responses among studies. In addition, there is no standardized dosage level among studies. For example, concentrations of 1 μg PHA in 2.5 μl PBS (phosphate-buffered saline; Navarro *et al.*, 2003) versus 2 μg PHA in 1 μl PBS (Smits *et al.*, 1999) have been used in birds. The source of PHA may also influence the results and it is therefore important to report the type of PHA used. In addition, it has become commonplace to use the absolute difference in thickness between post and pre-injection rather than a ratio. However, this linear measure does not control for differences in body size among individuals. It is likely that many patterns could be due to differences in size among individuals rather than actual immune activity if body size is not included as a covariate in the analysis. It should be noted that CMI responses are more complicated than a response to a single mitogen, but few studies have attempted to assess CMI using other techniques or even with other mitogens.

Humoral immune responses are typically evaluated via antibody production to novel antigens, which are usually mammalian erythrocytes (often SRBC) or inactivated vaccines, such as diphtheria–tetanus toxoid or inactivated NDV vaccines. Before addressing concerns and issues associated with this technique, it is relevant to point out that this approach has yielded many important and interesting results. Given the concerns detailed below, the fact that measures of immune response are consistent with predictions based on life history theory suggests that responses to novel antigens are reasonably good measures of immune performance.

However, these tests are not without their drawbacks. One reason why studies vary in their results comparing life history and immunology is that not all antigens stimulate the humoral immune system in similar ways. For example, Raberg and Stjernman (2003) found different patterns of natural selection on antibody response in response to diphtheria versus tetanus in blue tits (*Parus caeruleus*: Passeriformes), a result also found in red-winged blackbirds (*Agelaius phoeniceus*: Passeriformes) (Westneat *et al.*, 2003). A validation study comparing correlations among a variety of different common antigens is clearly needed. The most important set of criticisms of using novel antigens and mitogens is that disease resistance is more complex than a response to a mitogen or an inert antigen may indicate. Comparative studies of responses against inert antigens versus pathogenic challenge are critically needed to validate the former (Adamo, 2004).

The third component of the vertebrate immune system is innate immunity, which as yet is not widely assessed in field studies. Given the costs of upregulating the immune system, it is likely that natural selection has favoured many species with optimal levels of innate immunity as an important first line of defence (Lochmiller and Deerenberg, 2000). An intermediate level of response may be the optimal strategy as balancing investment in immune threats versus other life history components will be favoured most by natural selection.

THE MAJOR HISTOCOMPATIBILITY COMPLEX

The major histocompatibility complex (MHC) forms an integral part of the immune system and has a key role in selection for disease resistance. The MHC of chickens has been well characterized and Kaufman *et al.* (1999; Chapter 8) proposed that the chicken MHC is minimal essential, in which one single MHC class I molecule is dominantly expressed. This hypothesis may not be a general model for the MHC of all avian species. The diversity and number of MHC genes varies widely among passerine species and more than one can be expressed in individual birds (Westerdahl *et al.*, 2000; Edwards and Dillon, 2004). Using cDNA analysis Westerdahl *et al.* (2000) found that at least 4 MHC class II β loci were transcribed in an individual great reed warbler (*Acrocephalus arundinaceus*: Passeriformes). Similar results were obtained by R. Yunis and K.A. Schat (unpublished observations) using American crows (*Corvus brachyrhynchos*: Passeriformes). Limited studies suggest that migratory species may have a higher number of MHC genes and greater polymorphism than the sedentary species due to the greater diversity of parasite exposure (Westerdahl *et al.*, 2005). Westerdahl *et al.* (2005) found that the presence of six or more MHC class I alleles was beneficial for the control of acute malaria

in the migratory great reed warbler. On the other hand, MHC diversity in house sparrows (*Passer domesticus*: Passeriformes), a non-migratory species, correlated with clutch size and fledging success (Bonneaud *et al.*, 2004). Comparison of MHC diversity and immune responses to SRBC and PHA in house sparrows revealed that the total number of MHC alleles did not influence the primary and secondary responses, but one specific allele (a172) was correlated with increased responses to secondary PHA stimulation and primary and secondary antibody responses to SRBC (Bonneaud *et al.*, 2005). A study with wild-caught captive house finches (*Carpodacus mexicanus*: Passeriformes) found that heterozygosity (at 12 non-MHC loci) was correlated with both resistance to mycoplasma and increased PHA responses (Hawley *et al.*, 2005). In addition to disease resistance, MHC has also been linked to mate selection by females although that hypothesis remains contentious. For example, female Seychelles warblers (*Acrocephalus sechellensis*: Passeriformes) had more extra-pair paternity matings when their social mates had low MHC diversity (Richardson *et al.*, 2005). In contrast, there was no relationship between extra-pair paternity matings and MHC diversity in great reed warblers (Westerdahl *et al.*, 2004).

DEVELOPMENT OF THE IMMUNE SYSTEM IN FREE-LIVING BIRDS

Ontogeny

In general, immune system development should reflect the integration of disease and other selective pressures, as well as developmental constraints. In avian species, development to sexual maturity varies along a gradient from precocial to altricial growth, with the majority of species showing altricial or semi-altricial growth. However, at present, our understanding of the ontogeny of the avian immune system comes from domesticated birds, which are precocial. Relatively less is known about the ontogeny of immune function in altricial birds (Apanius, 1998). Altricial growth differs significantly from precocial growth in the mode and rate of development, particularly in the allometry of morphological development and the timing of thermoregulation (Starck and Ricklefs, 1998). Differences in immunological development between altricial and precocial birds may be due to differing developmental constraints as well as due to selection pressures associated with differing levels of disease exposure. For example, altricial birds may suffer more from parasites that take advantage of the low mobility of nestlings, such as hen fleas and blood-sucking fly larvae. Extrapolating knowledge from domesticated birds to free-living altricial species should be done with caution due to these marked developmental differences, as well as because of differences in body size and evolutionary history.

Most of our knowledge of the development of the immune system in birds comes from extensive work with domesticated species. However, research conducted with free-living species suggests that the ontogeny of the immune system is similar in free-living and domesticated species. For example, increases in serum IgY concentrations in the semi-altricial Leach's storm petrel and semi-altricial American kestrel (*Falco sparverius*: Falconiformes) are similar to those reported in the precocial domesticated chicken and duck (Apanius, 1998). Serum IgY levels did not differ between captive and free-living kestrels, suggesting that work on some wild-caught species kept in captivity may provide insight into processes occurring in nature. Serum IgY concentrations in 30- to 55-day-old storm petrel chicks (60-day nestling period) averaged 2.3 mg/ml, a range reported in poultry (Apanius, 1998).

While few field studies have been specifically designed to examine ontogeny of the immune system, it is clear that young birds can produce robust CMI responses to PHA (Brinkhof *et al.*, 1999; Hõrak *et al.*, 1999; Ardia, 2005a; Haussmann *et al.*, 2005). However, similar to domesticated birds, nestling free-living birds demonstrate a limited ability to produce an effective humoral immune response. In work done with the altricial rock dove (*Columba livia*: Columbiformes), B-cell differentiation occurred in a similar time frame as reported for precocial species (Apanius, 1998). For example, 2- to 6-day-old squabs are unable to produce a humoral

immune response in contrast to 7–10 day chicks; the switch from IgM to IgY over this development period supports the hypothesis that individuals were actually developing an antibody population, rather than using parentally derived antibodies (Apanius, 1998).

The most interesting finding on the comparative ontogeny of avian immune systems is that surgical bursectomy of pigeons does not limit future antibody production. This is in contrast to findings with poultry, where bursectomy of young birds leads to limited humoral immune responses as adults. Retained antibody production occurs even though pigeons show similar development of the bursa of Fabricius as reported for chickens. The bursa increases in size from hatching and reaches maximal size at 3–4 months of age (Selvaraj and Pitchappan, 1988). This important difference suggests more rapid B-cell differentiation in the altricial pigeon and may indicate an important difference in life history strategies (and thus natural selection pressures) between species. However, this is a single study performed with captive birds and needs to be replicated in the wild and across a range of species.

In one of the few studies done on juvenile free-living passerine birds, western bluebirds (*Sialia mexicana*: Passeriformes) were challenged with SRBC or killed NDV and orally dosed with different amounts of lead shot and examined for PHA responses (Fair and Myers, 2002). Lead, an important environmental immunotoxicant, can cause a shift from Th1 to Th2 responses especially during early developmental stages (Dietert *et al.*, 2004). There was no effect on the immune parameters except that the PHA response was reduced in the high lead treatment group. Interestingly, the chicks challenged with NDV or SRBC had a significantly higher survival rate while committing resources to antibody development (Fair and Myers, 2002). Others have found that pre-fledging passerine birds have a limited ability to produce humoral immune responses (e.g. tree swallow, *Tachycineta bicolor*: Passeriformes, D.R. Ardia, unpublished data).

One possible explanation for differences in B-cell development across species comes from a comparative study indicating that species with greater parasite exposure as adults had longer *in ovo* development periods (Ricklefs, 1992). In particular, species with long incubation periods had a lower prevalence of haematozoan blood parasites as adults. This lengthened incubation period (and thus embryonic development period) was hypothesized to lead to greater maturation of the immune system, particularly due to a greater number of cycles of proliferation of stem cells into idiotypes. In many species, new idiotypes are not generated after hatching, so longer development periods would appear to generate greater idiotype diversity (Ricklefs, 1992). Domesticated species of birds have relatively short incubation periods for their body size, thus their limited early B-cell development may not reflect the selection pressures facing other bird groups. However, few studies have reported early B-cell development in passerines, so this comparative hypothesis remains untested.

Parental Transmission of Antibodies

Mothers may influence the immune system of their offspring in many ways beyond direct genetic contributions. In birds, hens directly transfer antibodies to their naïve offspring, usually in the eggs, but in some species also to nestlings. These maternal contributions to offspring immunity are important because (1) they may provide the primary form of humoral immune defence early in development, (2) they may influence offspring growth rates both by directly stimulating cell-surface receptors involved in growth and/or by reducing the energetic cost of immune activity and (3) they may stimulate antibody production (Grindstaff *et al.*, 2003). In most cases, females transfer immunoglobulin as IgY in the yolk, however in species that provide crop milk (e.g. pigeons: Columbiformes) parents transfer serum IgA to their offspring (Goudswaard *et al.*, 1979). In non-poultry species examined in captivity, the duration of the period of passive immunity varies based on the antibody titres against relevant antigens in the female at time of egg production. Because the half-life of antibodies is probably constant across species, effective passive immunity will probably be present between 2 and 4 weeks after hatching based on data from chickens.

Maternal transmission of antibodies is beneficial for protection against pathogens early after hatching, but maternal antibodies may also prevent stimulation of the neonatal immune system. Within a species, differences among individuals in exposure, antibody production and transfer may occur due to a variety of factors, including social environment, prior disease exposure, food resources and parental condition (reviewed in Grindstaff *et al.*, 2003). One example of adaptive variation among individuals in transfer of maternal antibodies is in the great tit (*Parus major*: Passeriformes). Adult females exposed to hen fleas (*Ceratophyllus gallinae*) have high levels of anti-flea antibodies, which are thus passed to their offspring through eggs, increasing nestling resistance to fleas (Heeb *et al.*, 1998). This context-dependent transmission of IgY antibodies functions well because of the high cost of antibody production (Grindstaff *et al.*, 2003). As limited data are currently available, future research should examine species-level differences in maternal antibody transmission in light of differences in selection pressure from parasites. For example, ground-foraging birds are hypothesized to face greater exposure to blood parasites than are seabirds, while seabirds suffer more from helminths encountered while eating fish (Apanius, 1998). Thus, the suite of specific antibodies transferred in each species should address likely threats. Along with immune system development, transmission of maternal antibodies is the area of ecoimmunology in greatest need of future study. In particular, more research is needed on the costs and benefits of maternal transmission, on whether species-level differences reflect adaptive responses to selection pressures, and how exposure to maternal antibodies affects offspring immune system development.

FACTORS CAUSING VARIATION IN IMMUNE RESPONSES

Age-Related Variation

Ecoimmunological approaches are concerned with variation among individuals, and one important source of variation among individuals is age. Differences in immune performance among age groups may occur due to differences in past parasite exposure, body condition and foraging performance. Accordingly, all field studies should, at a minimum, control for age statistically and experimentally when examining responses. In addition, age differences may represent important life history differences among individuals within a species. For example, senescence, the progressive loss of function with age, may occur both due to constraints of ageing, as well as an adaptive strategy of downregulating investment in factors affecting long-term survival (e.g. the immune system) to increase investment in reproduction.

A study explicitly examining immunosenescence in three species (zebra finch *Taenopygia guttata*: Passeriformes, tree swallow, and Leach's storm petrel) found that individuals recently reaching adulthood consistently maintained stronger cutaneous CMI responses to PHA than older birds (Fig. 22.1; Haussmann *et al.*, 2005). Interestingly, immune response decreased more with age in the shorter-lived tree swallow and zebra finch than in the longer-lived storm petrel. These results argue strongly for more studies comparing species that differ in longevity, particularly among closely related species, in order to remove confounding differences of evolutionary history. Parrots (Psittaciformes) may be an excellent model group for asking these questions as they are generally long-lived but show considerable variation in longevity among species.

Age-related changes in humoral immune responses follow similar patterns of decreasing immune performance with age. Older (5–6 years old) collared flycatcher (*Ficedula albicollis*: Passeriformes) females showed a decline in antibody production to SRBC relative to younger individuals (Cichón *et al.*, 2003). Similarly, 1-year-old barn swallows (*Hirundo rustica*: Passeriformes) produced higher levels of primary and secondary antibodies to inactivated NDV than 3-year-old birds (Saino *et al.*, 2003). Both studies compared individuals of different ages at a single-time point; an alternative explanation is that individuals with strong immune responses have lower survival, leading to older individuals showing a lower average level of immune activity. In addition, these studies were conducted with relatively short-lived species; work done

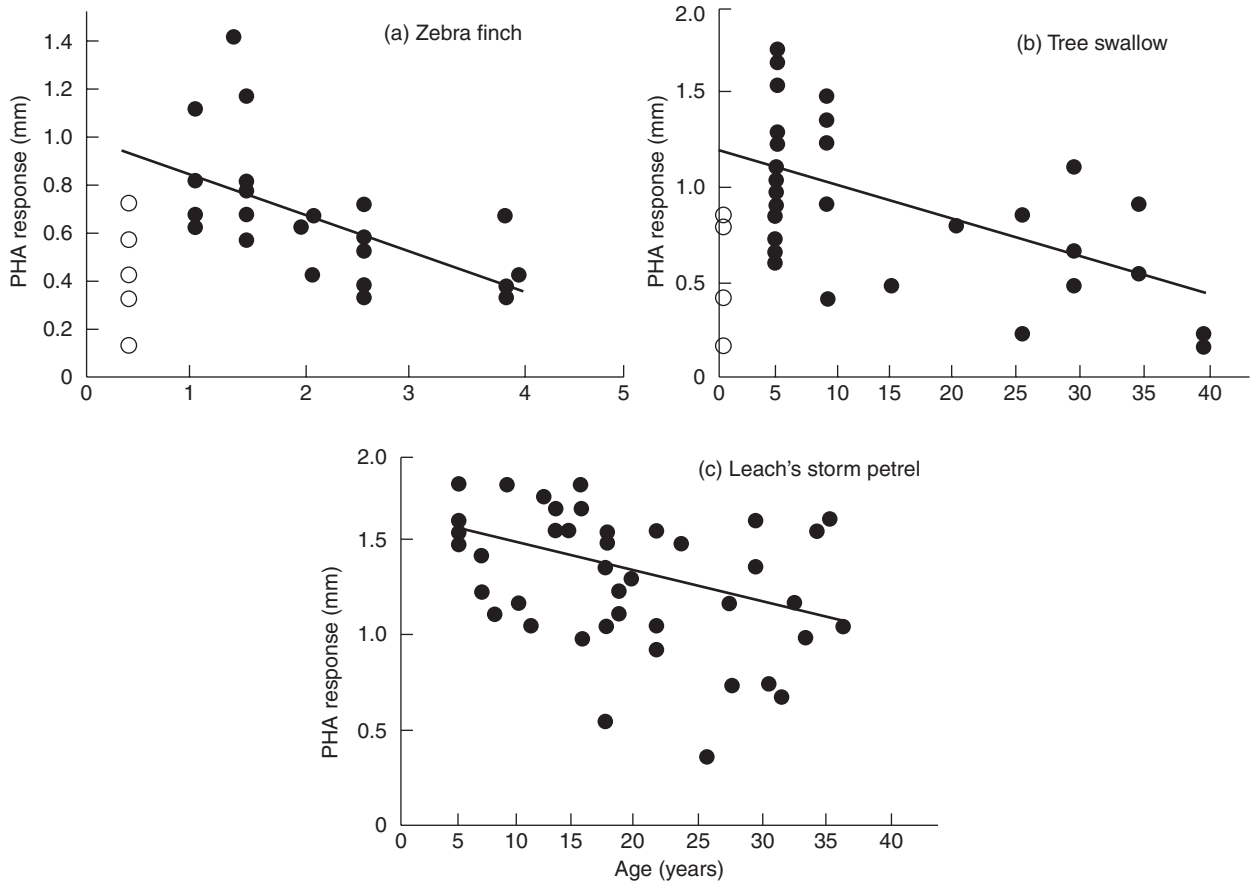

FIGURE 22.1 CMI response to PHA as a function of age for three species of birds. The lines are the least-squares regressions through the data in (a) zebra finches [slope = −0.153±0.04 (SE)]; (b) tree swallows [slope = −0.107±0.03 (SE)]; and (c) Leach's storm petrels [slope = −0.016±0.01 (SE)], for breeding adults (filled circle) and immature birds (open circle). Data from immature birds were not included in the regression analysis. Reprinted from Haussmann *et al.* (2005).

with the long-lived common tern (lifespan up to 23+ years) (*Sterna hirundo*: Passeriformes) found no steady decline in IgY levels with age (Apanius and Nisbet, 2003). The logical next step is to examine changes in immune performance in known individuals over time. In addition, more detailed work is needed on the mechanism that may underlie age-related changes, such as differences in lymphocyte proliferation, changes in antigen recognition or higher levels of autoimmunity, all leading to the overall consequence of decreased overall performance.

Condition and Nutrition

The central causes of the considerable variation observed in field studies of immune function are differences in condition and nutrition among individuals, in part because the vertebrate immune response is reliant upon significant supplies of proteins and amino acids (Klasing, 1998). Immune responses can affect growth (or be affected by growth) through two pathways: (1) reallocation of limited resources or (2) through systemic physiological controls on metabolic and developmental pathways. These complex interactions explain, at least in part, the variability in immune responses in field studies. In general, studies explicitly examining variation find that food intake, parental quality and maternal effects exert the strongest influence. The importance of dietary factors and immune responses is also discussed in Chapter 17.

Because of the difficulty of consistently recapturing adults, most studies examining the effect of condition or nutrition on immune responses have focused on nestlings. The most robust experimental design is a partial cross-fostering manipulation, where nestlings are exchanged among nests, leading each nest to contain a mix of nestlings, both related and not. This design helps to separate the effect of rearing conditions from common origin. Most cross-fostering studies have reported an influence of both common origin and common rearing environment on CMI responses to PHA, with the stronger effect appearing to be environmental conditions (Brinkhof *et al.*, 1999; Hõrak *et al.*, 1999; Ardia, 2005a). For example, a partial cross-fostering study in great tits found that common rearing environment explained the plurality of variation in PHA responses, mostly through its influence on nestling body mass (Brinkhof *et al.*, 1999). A sophisticated statistical analysis examining correlations among the many variables influencing nestling immune responses found that the strongest influence was a positive relationship with brood size and a negative relationship with nestling condition. Both variables reflect direct resource limitation and parental quality (e.g. genetics, maternal effects and trace nutrient delivery) (Westneat *et al.*, 2004). Unfortunately, much less is known about the role of common origin versus common rearing environment in humoral immune responses because of limited immunological development in nestlings. A food supplementation study in serins (*Serinus serinus*: Passeriformes) found that individuals with increased food intake produced stronger humoral responses (Hoi-Leitner *et al.*, 2001).

Studies examining variation in adults also find a strong role of resource levels on the immune response. For example, adult tree swallows feeding nestlings during cold conditions with low food abundance mounted weaker CMI responses to PHA (Lifjeld *et al.*, 2002), suggesting that short-term differences in environmental conditions can affect cell-mediated responses. Adult house sparrows produced stronger PHA responses at night than during the day, suggesting that lower energetic demands at night (due to lower activity levels) were linked with higher PHA responses (Navarro *et al.*, 2003). In addition, house sparrows with higher residual body mass (i.e. greater resource stores) showed stronger PHA responses. In another study using house sparrows, individuals exposed to a predator downregulated the PHA responses, which was likely due to increased levels of stress hormones or adaptive resource allocation (Navarro *et al.*, 2004). These studies indicate that CMI responses to PHA can be highly dynamic and vary not only among individuals, but also within individuals due to differences in resource availability. This strongly indicates that differences in quality among individuals are a central source of variation.

That individuals differ in their ability to survive and reproduce is one of the central tenets of natural selection theory (Darwin, 1859). Immune responses have been found to vary with individual quality or identity, such as when immune responses are greater in larger natural broods (Tella *et al.*, 2000; Westneat *et al.*, 2004; Ardia, 2005a) or as a function of individual variation. For example, early breeding (presumably higher-quality) female tree swallows are better able to deal with tradeoffs between immunological self-maintenance and offspring quality and produce stronger PHA and humoral SRBC immune responses than do later-laying females (Fig. 22.2; Ardia, 2005b). Similarly, common terns with high reproductive performance have higher levels of serum IgY than poorer performers (Apanius and Nisbet, 2006). In house finches and jungle fowl (*Gallus gallus*: Galliformes), individuals of high dominance status, another indicator of quality, produce the strongest immune responses to injections with PHA and/or SRBC (Zuk and Johnsen, 2000; Hawley *et al.*, 2006).

Individual differences may be due to both greater ability to gather resources and differences in genetic background. Most studies find a significant influence of common origin on nestling immune responses, indicating a heritable component of immune activity (e.g. Brinkhof *et al.*, 1999; Ardia, 2005a), which is not surprising in view of genetic selection for increased immune responses and resistance to diseases in chickens (Schat and Davies, 2000). However, work on inheritance of immune activity is in its infancy in free-living birds. As a consequence, little is known about variation in the strength of natural selection. Moreover, the relative effects of additive genetic variance (i.e. genetic variance associated with the average effects of substituting one allele for another) on immune function vary depending on the strength of natural selection.

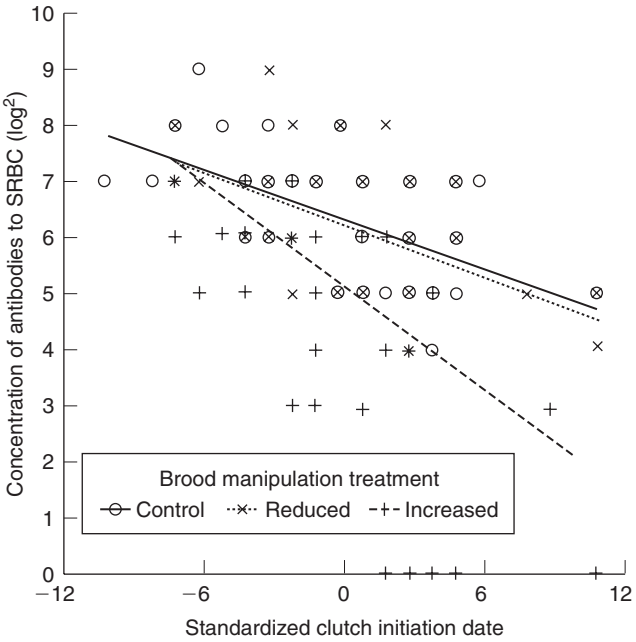

FIGURE 22.2 Antibody production after humoral immune challenge with SRBC in female tree swallows (*Tachycineta bicolor*) subjected to a brood size manipulation as a function of clutch initiation date. Date of clutch initiation is standardized within each year to mean = 0. Reprinted from Ardia (2005b).

Work comparing heritability of immune responses among populations finds spatial variation, with some sites showing significant heritability and others no apparent role of additive genetic variation (Ardia and Rice, 2006).

Parasite Exposure

Parasites can have two main effects on immune responses: (1) stimulating immune activity and (2) reducing resource reserves. These two influences may have counteracting effects. Parasitism can stimulate higher immune activity relative to unexposed individuals, but due to the debilitating influences of parasites, a reduction in resource reserves could occur that leads to reduced immune responses. Because of these counteracting effects, it is important to demonstrate that parasites actually stimulate the immune system. Exposure to the intestinal nematode (*Ascaridia galli*) in jungle fowl caused an increase in granulocyte levels, but a decrease in CMI responses (Johnsen and Zuk, 1999). A similar study in pied flycatchers (*Ficedula hypoleuca*: Passeriformes) found that *Haemoproteus* exposure increased IgY levels, but also led to reduced PHA responses (Morales *et al.*, 2004). These results indicate that exposure to parasites stimulates an immune response, but low immune activity may reflect a reduced ability to mount a strong immune response.

Due to these counteracting effects, it is not surprising that studies finding significant relationships between parasites and immune responses vary in their conclusions. In a well-designed study using a fumigant, the effect of exposure to the haematophagous parasitic house martin bug *Oeciacus hirundinis* was examined on the immune responses in nestling house martins (*Delichon urbica*: Passeriformes) (Christe *et al.*, 2000). Individuals exposed to ectoparasites showed lower PHA responses than individuals who had ectoparasite loads controlled through fumigation, indicating a greater role of resource depletion relative to immune stimulation. Similarly, house sparrows with high levels of *Haemoproteus* blood parasites showed lower PHA responses, due almost exclusively to loss of body mass (Navarro *et al.*, 2003). Experimental

increase of protein intake in house sparrows led to an increase in PHA responses and a reduction in *Haemoproteus* infection (González *et al.*, 1999). In addition, variation in the influence of workload (reflected in feeding effort) on blood parasites (*Trypanosoma, Haemoproteus*) in pied flycatchers leads to yearly differences, indicating that the effect of parasitism is heavily dependent on environmental conditions (Sanz *et al.*, 2002). While these studies find a significant effect of parasite load on immune activity, others have found no effect at all when examining ectoparasite levels (Brinkhof *et al.*, 1999; Tschirren *et al.*, 2003; Ardia, 2005a). Results of studies assessing the role of ectoparasites on the immune response should be viewed within the context of counteracting pressures.

IMMUNE FUNCTION AS A LIFE HISTORY TRAIT

Costs of Mounting Immune Responses

For immune function to be viewed in a life history context, investment in immune activity must tradeoff with life history components (Norris and Evans, 2000). In other words, investment in a competing component of life history, such as growth or reproduction, should lead to decreased immune response or vice versa. The first study to show conclusively that reduced immune functions during breeding were due to adaptive reallocation of resources was with captive zebra finches (Deerenberg *et al.*, 1997). Breeding females who raised enlarged broods showed lower antibody responsiveness to SRBC than did control females. The downregulation appeared to be due to increased workloads because females trained to increase daily activity levels showed similar levels of immunosuppression (Deerenberg *et al.*, 1997). This central outcome of tradeoffs between reproductive effort and immune responses has also been reported in free-living birds in many different contexts (Lochmiller and Deerenberg, 2000; Norris and Evans, 2000). Most studies have examined whether a manipulation of reproductive effort (typically a brood size manipulation) leads to decreased immune responses. A range of studies have found that increased work load leads to reduced responses to PHA (Moreno *et al.*, 1999; Ardia, 2005c). Conversely, others have investigated whether challenge to the immune system leads to reduced reproductive effort. For example, blue tits mounting humoral immune responses to diphtheria–tetanus toxoid reduced feeding activity (Raberg *et al.*, 2000). Another indication of the tradeoff between reproductive effort and immune activity is that increased effort often leads to increased parasite susceptibility (Gustafsson *et al.*, 1994; Norris *et al.*, 1994; Nordling *et al.*, 1998). Female collared flycatchers inoculated with inactivated NDV reduced reproductive effort and showed increased susceptibility to *Haemoproteus*. In addition, females with depressed immune responses also showed decreased resistance to parasites (Nordling *et al.*, 1998).

Because of the important role that body condition plays in influencing the immune response, it is important to make the distinction between direct effects on immune responses due to evolved tradeoffs or indirect effects of condition on immune responses (Sheldon and Verhulst, 1996). While both pathways show a tradeoff between resource allocation and immune response, it may be important to consider more complicated interactions between other components of life histories and immune performance. For example, moulting house sparrows with rapid feather growth showed weaker PHA responses (Martin, 2005). In general, more work is needed to examine other life history traits (e.g. migration, courtship and overwintering) to determine the extent of tradeoffs and the level of individual variation.

A central assumption of a life history perspective on immune function is that high reproductive effort leads to nonadaptive immunosuppression caused by resource allocation and the detrimental effects of increased free radicals (Sheldon and Verhulst, 1996; Norris and Evans, 2000). Immunosuppression stemming from the reallocation of nutrients away from self-maintenance to reproductive effort may be adaptive in short-lived birds because immunosuppression increases parent fitness by maximizing offspring. In longer-lived birds, sustained allocation of nutrients to immune function during reproduction may be adaptive because the absence

of immunosuppression increases parent fitness by prolonging reproductive lifespan (Apanius and Nisbet, 2006).

An untested but compelling alternative hypothesis is that stress-related immunosuppression is actually adaptive (Raberg *et al.*, 1998). Raberg *et al.* (1998) hypothesize that immune activity entails limited energetic costs, and thus is not constrained by resources. Consequently, they propose that stress-related immunosuppression is beneficial in order to reduce risk of autoimmune reactions occurring with a hyperactivated immune system; immunosuppression to avoid autoimmunity is an alternative adaptive explanation without regard to longevity. Recent work suggests that immune responses may be more energetically costly than envisaged by Raberg *et al.* (1998; reviewed in Lochmiller and Deerenberg, 2000). These two competing hypotheses to explain stress-induced immunosuppression are not necessarily mutually exclusive. It is likely that the patterns observed in nature represent a mix of strategic downregulation, as well as nonadaptive resource limitation. Future work is needed to better elucidate the conditions, such as the probability and severity of disease exposure, when downregulation is adaptive.

The second central assumption of examining immune function in a life history context is that levels of immune response should be linked with changes in reproduction. Few studies have been able to directly examine this relationship in free-living birds. The most convincing approach is to experimentally manipulate parasite levels (and thus decrease investment in immunity) to examine the effect on reproductive success. A medication experiment with primaquine on house martins (*Delichon urbica*: Passeriformes) revealed that treated individuals free from malarial parasites had increased fledging success, suggesting that energy diverted from fighting infection was invested in offspring (Marzal *et al.*, 2005). In addition, house sparrows injected with lipopolysaccharide (LPS) had reduced reproductive success, in part due to decreases in feeding effort (Bonneaud *et al.*, 2003). However, LPS exerts pleiotropic effects including induction of fever, which could have affected feeding behaviour.

A third critical assumption is that levels of immune functions correlate with survival. Some immune responses are correlated with increased probability of survival; for example, high total immunoglobulin levels in nestling house martins predicted survival and reflected in recapture rates but, interestingly, PHA responses did not (Christe *et al.*, 2001). However, studies in pied flycatchers and blue tits found that PHA responses in nestlings predicted return rates in the year following fledging better than more conventional predictors such as timing of fledging and body mass (Cichon and Dubiec, 2005; Moreno *et al.*, 2005).

In adults, there is evidence that high levels of immune responses are also correlated with higher survival. For example, female tree swallows producing strong secondary immune responses to SRBC had higher return rates (Ardia *et al.*, 2003). Conversely, female collared flycatchers with reduced NDV responses were more susceptible to *Haemoproteus* and had increased mortality rates (Nordling *et al.*, 1998), while barn swallows with strong humoral responses to SRBC had increased survival rates (Saino *et al.*, 1997). But, given that immune responses are costly in resources, there may be circumstances where intermediate immune responses may be beneficial compared to high or low responses. Levels of immune response during winter in blue tits showed evidence of both directional selection, with high responses to secondary stimulation with tetanus toxoid, and stabilizing natural selection with intermediate responses to diphtheria–toxoid (Fig. 22.3; Raberg and Stjernman, 2003). A key next step is to apply these results to studies during the breeding season, as there should be differences between nonbreeding and breeding due to maximal work load and seasonal variation in both immune system threats and stress-related endocrine–immune interactions. However, it does suggest that high levels of immune response may not always be the best allocation strategy in terms of lifetime survival and reproduction.

All of these studies are predicated on the assumption that immune activity entails energetic costs. However, only a few studies have directly examined the energetic costs of immune response in free-living birds. Wild-caught house sparrows producing CMI responses to PHA stimulation showed significantly elevated resting metabolic rates (RMR) relative to controls inoculated with saline, with a total daily energy cost of approximately 4.20 kJ per day (29%

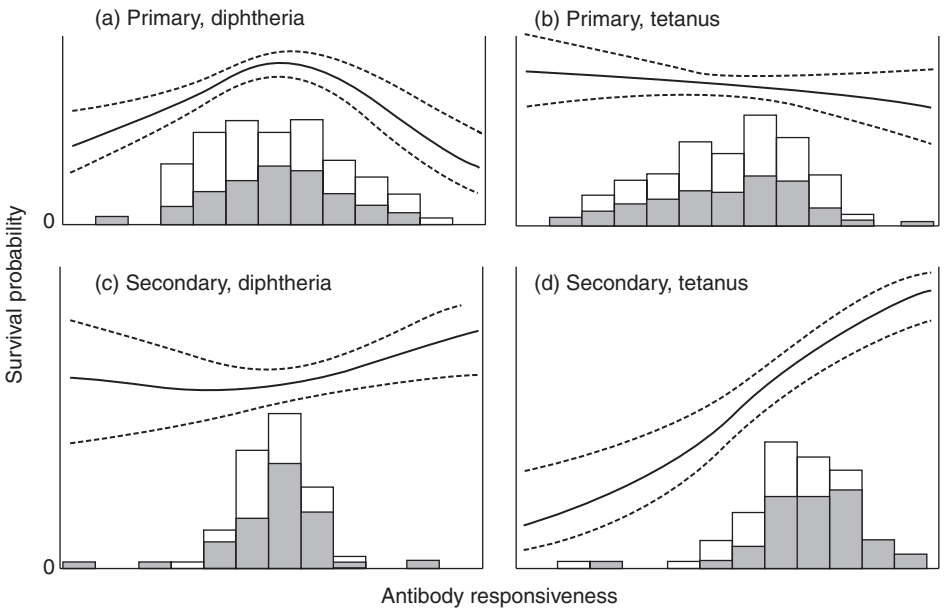

FIGURE 22.3 Survival probability of wintering blue tits (*P. caeruleus*) as a function of antibody responsiveness for primary and secondary responses to diphtheria and tetanus toxoids. The solid lines are fitness functions as estimated by the cubic spline technique and dashed lines are standard errors. *j* is the quadratic selection differential, and *i* is the linear selection differential. The *p*-values determined by logistic regression. The histograms represent the frequency distribution before (open 1 shaded) and after selection (shaded). Reprinted from Raberg and Stjernman (2003).

RMR), which is equivalent to the cost of production of half of an egg (8.23 kJ/egg) (Martin *et al.*, 2003). In field studies, antibody response to SRBC inoculation led to a 9% increase in basal metabolic rate (BMR) in great tits relative to saline-injected controls (Ots *et al.*, 2001). However, antibody response to diphtheria–tetanus toxoid in blue tits kept in aviaries did not significantly elevate BMR (Svensson *et al.*, 1998). While it is difficult to generalize from these studies, it appears that CMI responses to PHA may entail greater energetic costs than humoral responses.

The experimental studies described here give a rough range of the direct and indirect costs of producing an immune response following antigenic challenge. While this is an important component of the cost of immunity, it is not a complete assessment, as it is not clear what is the cost of maintaining a well-functioning immune system (Lochmiller and Deerenberg, 2000). In particular, there is limited knowledge of the energetic and nutrient costs of the maintenance of lymphoid tissue, removing free radicals and the turnover of leukocytes. More work is needed on the basic mechanistic aspects of this question, which should greatly enhance the ability to test evolutionary hypotheses about species differences.

Links with Male Secondary Sexual Characters

If investments in immune functions are costly, not all individuals should be equally able to maintain similar levels of immunity. These differences should lead to predictable variation in immune performance reflecting condition-dependent differences among individuals. Thus, the role of immunity in life histories should be reflected in condition-dependent secondary sexual characters. It is hypothesized that animals choose mates based on disease resistance (due to the strong selection pressure of parasites) and that as a consequence many secondary sexual characters are linked to immune responsiveness. If secondary sexual signals are useful indicators for mate selection, there must be individual variation in the signal. In other words,

all males seek to produce the maximum signal, but only a small number are able to do so. Since Darwin, researchers have believed that sexual dimorphism in bright colour patterns is due to sexual selection (i.e. differential mating success). Our understanding of the underlying mechanism of what females gain by mating with bright males has varied over the years. The first link between secondary sexual characters and immune function was proposed by Hamilton and Zuk (1982). They hypothesized that bright males were signalling a greater health state and they reported that bright colours were most common in species with high levels of blood parasitism (Hamilton and Zuk, 1982; Folstad and Karter, 1992). However, their initial hypothesis that bright colours signal a genetic link with parasite resistance has proved difficult to test, thus most studies now focus on condition-dependent immune responses and sexually selected traits.

High immune performance in offspring may benefit the mating females, but this is a difficult trait to assess during courtship; therefore, other more easily assessed traits may have evolved that are correlated with immune performance. For example, male barn swallows with naturally long tails (a preferred ornamental character in European barn swallows) also had a higher immune response to SRBC, while short-tailed males with experimentally elongated tails had a reduced immune response (Saino *et al.*, 1997). Additional evidence of the link between immune activity and a secondary sexual trait is the effect of malaria (*Plasmodium relictum*) on song learning in canaries (*Serinus canaria*: Passeriformes). Infection with malaria reduced development of the high vocal centre (HVC) song nucleus in the brain (Spencer *et al.*, 2005). These experimental results support the finding that species of birds with high levels of song complexity also had high levels of PHA responses (Garamszegi *et al.*, 2003), though more work is needed to separate the pathogenic effects of malaria from direct immunological stimulation. The role of carotenoids in mediating links between sexually selected coloration and immune responses has been elucidated in a number of experiments. Carotenoid-supplemented male zebra finches showed increased bill colour, PHA responses, and mating success (Blount *et al.*, 2003b). In a separate study, supplementation with carotenoid increased bill colour and both PHA responses and SRBC responses, as well as actual blood carotenoid levels (McGraw and Ardia, 2003).

However, other studies have investigated links between condition, sexual signals and immune response with mixed results (Birkhead *et al.*, 1998; González *et al.*, 1999). For example, in an extensive study in red-winged blackbirds, there was no correlation between humoral response to diphtheria–tetanus toxoid and male colour traits or reproductive success (Westneat *et al.*, 2003). There is also evidence that there may be differences among the arms of the immune system in their role in sexual selection. For example, responses to PHA correlated with train length (a sexually selected trait) in male blue peacocks (*Pavo cristatus*: Galliformes), but humoral immune response to SRBC did not (Møller and Petrie, 2002). In European blackbirds (*Turdus merula*: Passeriformes) SRBC response had no correlation with bright bill colour in males (a sexually selected trait) but PHA response had (Faivre *et al.*, 2003).

Because of general differences among males and females, different selection pressures often lead to general differences between the sexes in immune response. This is especially true in species with marked sexual dimorphism, such as ruffs (*Philomachus pugnax*: Charadriiformes) where males showed greater variance in PHA response than females (Lozano and Lank, 2003). However, in species with less marked size dimorphism (e.g. tree swallows), differences in PHA responses are not frequently reported (Lifjeld *et al.*, 2002). In colour-dimorphic species, males may show lower levels of immune response, such as in nestling great tits (Tschirren *et al.*, 2003). However, these differences may vary depending on the arms of the immune system stimulated. Male zebra finches produced a stronger humoral response to SRBC but a lower cell-mediated response to PHA than did females (McGraw and Ardia, 2005). Males may modulate immune function differently; house sparrows show a seasonal depression under captive conditions even when not showing raised testosterone levels (Greenman *et al.*, 2005). This difference in seasonal changes is also found in barn swallows, where males show less decline over the season relative to females (Saino *et al.*, 2003) and in ruffs, where males have higher non-breeding PHA responses than do females (Lozano and Lank, 2003).

IMMUNE FUNCTION IN AN EVOLUTIONARY CONTEXT

While much of ecoimmunology seeks to examine variation among individuals within a species, the selection pressures and tradeoffs associated with investment in immune function can also cause differences at the species level. It is important to make the distinction between within and among species interactions in parasite pressure and immunocompetence. For example, within a species, in general, individuals with strong immune activity have lower parasite-induced mortality. However, among species, greater levels of parasite-induced mortality will select for higher levels of immune function, even if the trend within the species is that high levels of mortality are correlated with low levels of immune performance (Martin *et al.*, 2001). Thus, an individual's level of immune function will reflect (1) species-level trends in the strength of natural selection from parasite exposure (causing greater investment in immune function relative to species with low levels of parasite pressure) and (2) individual-level differences.

Recently, investigations have focused on the role of immune function in influencing larger patterns of life history variation among bird species (Ricklefs and Wikelski, 2002). In general, birds in tropical regions are believed to have higher yearly survival rates. Tropical birds show a suite of life history adaptations that indicate a lower investment per breeding attempt and greater investment in self-maintenance, consistent with a longer lifespan. Researchers have focused on assessing immune performance as both an indicator of immunological self-maintenance and a selective force driving larger life history patterns (Ricklefs and Wikelski, 2002; Martin *et al.*, 2004; Ardia, 2005c). A brood size manipulation experiment across the geographic distribution range of tree swallows found population-level differences in investment in cell-mediated and humoral immune functions consistent with life history predictions. A northern population increased feeding effort and reduced immune responses, while a southern population did not (Ardia, 2005c). However, these differences may be due to phenotypic plasticity, whereby all individuals retain the ability to modulate immune activity and population-level differences are due simply to responses to environmental conditions. In an elegant common-garden experiment comparing tropical and temperate populations of house sparrows (as a surrogate for species-level differences), tropical house sparrows raised under the same conditions as temperate individuals showed different patterns of PHA responses (Fig. 22.4; Martin *et al.*, 2004). Tropical individuals maintained similar levels of immune function throughout the year, while temperate individuals showed a peak during the breeding season. In addition, temperate individuals had higher immune responses during the breeding and non-breeding season, while tropical individuals had higher immune responses only during the early breeding season (Martin *et al.*, 2004). Underlying differences between populations and across species may be due to differences in endocrine–immune system interactions, where in most situations high levels of stress hormones, such as corticosterone and suppress immune responses. In locations where the probability of exposure to parasites is both high and seasonally invariant, individuals may have evolved a lower sensitivity to the immunosuppressive effects of stress hormones. Temperate house sparrows experimentally implanted with corticosterone showed reduced immune responses while tropical house sparrows did not (Martin *et al.*, 2005). This suggests that tropical individuals have evolved different physiological pathways for addressing the selection pressures associated with greater immune threats.

Differences among species in immune abilities may lead to differences in their ecological and evolutionary success. For example, one major reason why introduced species thrive in novel environments is lack of natural parasites (Lee and Klasing, 2004). Thus, differences in immune performance among species may help to explain why some species are more successful as invaders than others, as well as help to explain differences in natural ranges among closely related species (Lee and Klasing, 2004). One hypothesized difference among species is that successful invaders will be less likely to mount costly inflammatory responses when encountering novel pathogens and thus will rely more on less costly immune responses, such as humoral responses (Lee and Klasing, 2004). Experimental work comparing the successful invader, the house sparrow, with its congener the tree sparrow (*Passer montanus*: Passeriformes) found that house

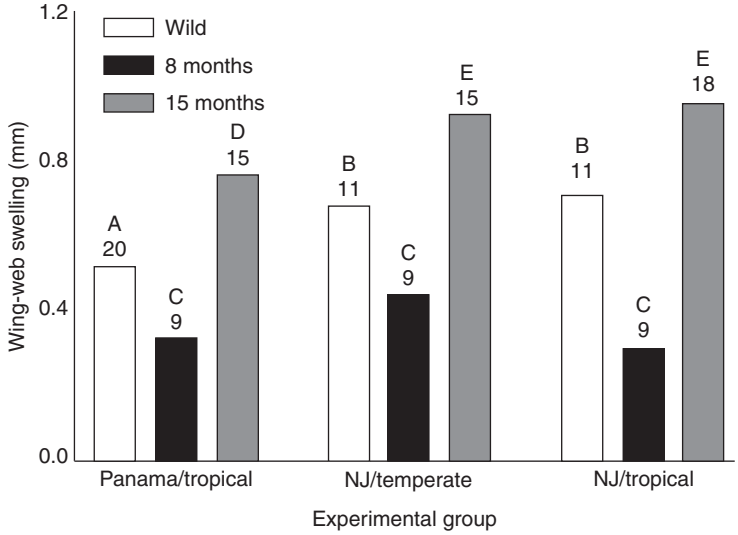

FIGURE 22.4 PHA-induced wing-web swelling during the non-breeding season in wild and common-garden-housed house sparrows from New Jersey, USA, and Colon, Panama. Open bars represent free-living values, solid bars represent 5-month captive values, and hatched bars represent 18-month captive values. Labels for experimental groups indicate latitude of origin and the climate in which they were held. Different letters above the bars indicate significant differences between groups ($p < 0.05$) based on a variety of statistical tests. Numbers indicate sample size. Histogram bars depict means. Reprinted from Martin *et al.* (2004).

sparrows showed less extensive inflammatory responses to LPS than did tree sparrows (Lee *et al.*, 2005). This difference may be relevant in view of potential challenges with Gram-negative bacteria, many of which have a high LPS content in their membranes.

These predictions highlight possible differences in the costs and benefits of the different arms of the acquired immune system, which suggest that extrapolating from one arm to a broader assessment of an individual's immunocompetence may ignore interactions among the different arms. Because of the role of immune system polarization, it may be difficult to produce strong cell-mediated and humoral immune response simultaneously (Graham, 2002). A study examining a wide diversity of avian orders found no relationship when comparing species pairs in their ability to produce responses in both arms simultaneously (Blount *et al.*, 2003a), though this study was limited by small sample sizes. Captive red jungle fowl with high IgY levels had low levels of cutaneous hypersensitivity response suggesting a condition-dependent tradeoff between cell-mediated and humoral immunity (Johnsen and Zuk, 1999). These results were supported with work done in the field. In a study comparing individual responses, individual tree swallows that produced strong humoral responses to SRBC were unable to also produce strong cell-mediated response to PHA (D.R. Ardia, unpublished data). These results suggest that more research is needed to identify the costs and benefits of allocation to different arms of the adaptive immune system, in particular, examining other immunochallenge agents.

PRIORITIES FOR FUTURE RESEARCH

Many solid contributions to evolutionary biology, life history theory and behavioural ecology have emerged from eco-immunological studies. However, as the field matures, it is entering a new phase where underlying assumptions need to be tested. In addition, due to the fragmentary

nature of studies, greater integration of results and interpretations is needed. Here, we suggest areas of future work that are most needed for the field of ecoimmunology to move forward:

1. *Better validation of methods used to measure immune activity*. Many of the standard techniques used to assess immune responses are older techniques used with poultry. However, compared to modern poultry techniques, they are relatively crude. More sophisticated laboratory techniques are badly needed that will allow comparison, in a controlled setting, of field measurements made from free-living birds, to determine how well ecoimmunological measures assess immune performance.
2. *Test the central assumption that immune response to an inert antigen predicts immune performance against actual disease threats*. In most studies, it is assumed that high levels of immune activity to a mitogenic or antigenic challenge indicate a high level of immune performance. However, it has recently become clear that variation among individuals and studies can be due to many factors. While conducting experiments comparing immune response to disease resistance is difficult, more work is needed in this critical area.
3. *Comparative immunological ontogeny*. There may be marked differences in the extent and rate of immunological development among avian species. In particular, different natural selection pressures can underlie patterns of variation. Systematic studies of immune system development across a range of avian species is needed to determine how the immune system interacts with other life history traits.
4. *The role of maternal antibody transmission*. Given the critical role of antibody transfer in influencing tradeoffs in offspring development, more research is needed on the costs and benefits of maternal transmission, and on how species-level differences reflect adaptive responses to selection pressures.

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APPENDIX 1

Genetic Stocks for Immunological Research

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INTRODUCTION
LINES WITH A DEFINED MHC
INBRED LINES
CONGENIC LINES
RANDOMBRED LINES
SELECTED LINES
GENERAL LINES
REFERENCES

INTRODUCTION

Currently available avian genetic resources for use in immunological research are listed in Tables A1.1–A1.6. Brief explanations for the general categories of lines listed in these tables are provided below. The main categories are based on specialized breeding schemes and genetic characteristics (inbred, congenic, randombred, selected) or selected characteristics, such as the major histocompatibility complex (MHC). Expanded explanations for the categories and further details on stocks can be found in Delany and Pisenti (1998) and Pisenti *et al*. (1999) which also provide additional references. Curator names and institutions are provided as known at the time of compilation of the tables; the references indicated for the lines were the suggestions by the curators. For line availability and exact details, the curators should be contacted.

LINES WITH A DEFINED MHC

A large number of poultry stocks useful for immunological research are in this category because they have been described and selected for particular MHC alleles. This is not a breeding category *per se*, except that the lines were originally selected on the basis of their haplotype. This needs to be verified on a regular basis to confirm that it contains the relevant MHC (Table A1.1).

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INBRED LINES

An inbred line is a genetic stock of highly related individuals, sharing in common their genetic sequence. A common breeding scheme to create highly inbred lines is full-sib mating for a period of 10 or more generations. The calculated inbreeding coefficient (*F*) expresses the relatedness of the individuals. The true level of inbreeding can be lower than that calculated, if one is selecting the "best" individuals for breeding, because heterozygous individuals may persist. Two generations of full-sib (brother × sister) mating results in an *F*-value of 0.375, which is considered the lower boundary for calling a stock inbred. When the *F*-value is this level the stock can be referred to as a "partially inbred line". Whereas stocks with *F*-values of 0.85–0.99 are referred to as "highly inbred" (Table A1.2).

CONGENIC LINES

Congenic lines differ from one another for only a small region of the genome, the "selected" gene or complex plus closely linked regions. Numerous chicken congenic lines exist that differ for their MHC haplotype while sharing the genetic background. Pairs of MHC congenic lines are created by crossing an inbred line with a MHC of interest (selected MHC) into a highly inbred "parent" line having a different MHC. The resulting MHC heterozygous progeny are backcrossed to the inbred parent line with continued selection for the heterozygous MHC combination, typically for a minimum of 10 generations. Thus, each successive generation gains more of the background parent genotype while the two MHC types and tightly linked genetic regions are retained through selection. Originally selection was accomplished by using the classic haemagglutination assay detecting B-G (MHC Class IV) alloantigens although now molecular sequences can be used for verification (see Fulton *et al*., 2006). Following the 10th generation backcross, the resulting heterozygous birds are crossed *inter se* and the resulting alternate homozygous birds for each MHC type are used as the founding population for a pair of MHC congenic lines. These share the same parent background (of the original inbred parent line) but differ for MHC type with one line having the donor or selected MHC and the other the original parent MHC type. Each line is perpetuated by within-line breeding and should be verified on a regular basis for MHC haplotype (Table A1.3).

RANDOMBRED LINES

Randombred stocks are unselected, closed populations established and maintained for the purpose of providing control stock for comparison to related "selected" lines. By unselected, it means that the dams and sires mated to create the next generation are not chosen for any specific phenotypic criteria, i.e. intentional pairing of breeders is avoided. Line maintenance typically requires relatively large numbers (150–200 birds) to minimize inbreeding (Table A1.4).

SELECTED LINES

Selection of mating pairs for breeding is based on specific criteria, often for quantitative traits governed by multiple genes. Breeders are selected for particular traits (phenotypes), e.g. resistance or susceptibility to disease, body size, egg traits and antibody response. Many selected lines have a "line bred" breeding scheme (versus "outbred", i.e., crossing with a different line) in that there is mating of those animals within a population exhibiting extreme high or low values for a desired trait (e.g. small versus large body size; Table A1.5).

GENERAL LINES

Lines in this category do not fit one specific genetical definition but are included because they have been found useful for immunologically related research. Often such stocks are closed breeding stocks, e.g. line bred with no intentional crossing between other unrelated lines (Table A1.6).

ACKNOWLEDGEMENTS

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TABLE A1.1 MHC-Defined Lines Useful for Immunological Research

Location (Curator)[a]	Stock Name[b]	Origin and History	Description/Notes[c]	References[d]
Cornell University (Schat, K.A.)	Cornell N2a	SCWL	MHC: B21, E-a alloantigen C2; highly resistant to MD; SPF	Bacon *et al.* (2001), Cole and Hutt (1973)
	Cornell P2a	SCWL	MHC: B19, E-a alloantigen C2; highly susceptible to MD; SPF	Bacon *et al.* (2001), Cole and Hutt (1973)
North Carolina State University (Ashwell, C.)	UNH 105 (transferred from University of New Hampshire 2007)	NH. Closed flock since 1981	Four MHC types in a closed flock: B22, B23, B24, B26; gene pool	
	UNH 193 (transferred from University of New Hampshire 2007)	SCWL × Ancona; Trisomic for MHC	MHC: B19. Mating trisomic parents produce disomic, trisomic and tetrasomic progeny	
Northern Illinois University (NIU) (Briles, W.E.)	NIU Female Breeder Parent Stock B19	Mixed Ancona derived from Ancona synthetic stocks homozygous for non-MHC system genes	MHC: B19 homozygotes for use as female parent in immunological challenges	
	NIU Female Breeder Parent Stock B2	Mixed Ancona derived from Ancona synthetic stocks homozygous for non-MHC system genes	MHC: B2 homozygotes for use as female parent in immunological challenges	
	NIU Female Breeder Parent Stock B5	Mixed Ancona derived from Ancona synthetic stocks homozygous for non-MHC system genes	MHC: B5 homozygotes for use as female parent in immunological challenges	
	NIU Bobwhites	From Mississippi State University in 1992	Northern Bobwhite quail with erythrocyte alloantigens; Various E-a	
	NIU Segregating Male Breeder Line	SCWL	MHC: B2/B5 or B19/B21; A4E1/A5E2; C2/C5; D1/D3; H1/H2; I2/I8; K2/K3; L1/L2; P1/P4; Various E-a.	
	NIU B haplotype recombinants	Mixed Ancona derived from Ancona synthetic stocks homozygous for non-MHC system genes	Recombinant B haplotypes: R1, R2, R3, R4, R5, R6, R7, R8, R9, R10, R11, R12; gene pool	

(*Continued*)

TABLE A1.1 (Continued)

Location (Curator)[a]	Stock Name[b]	Origin and History	Description/Notes[c]	References[d]
Northern Illinois University (Briles, W.E.)	NIU Male Breeder Alloantigen Reservoir	SCWL. Segregating for erythrocyte and non-erythrocyte alloantigens	Pool of cell surface erythrocyte alloantigen (# alleles): A(8), MHC: B(40), C(8), D(5), E(10), H(2), I(7), K(2), L(2), P(10), R(2); leukocyte alloantigens: M(5), N(2), Q(3), T(3), U(4), W(2), Z(2)	
	NIU B haplotypes	Mixed Ancona derived from Ancona synthetic stocks homozygous for non-MHC genes,	Pool of MHC B haplotypes: 1, 3, 4, 6, 8, 10, 11, 12, 13, 14, 15, 17, 22, 23, 24, 26, 30, 31, 32, 33, 24r1, 2r1, 2r2, 2r3, 21r1, 21r2, 2r4, 2r5, 24r2, 24r3, 21r6, 8r1; gene pool of various MHC (B) haplotypes, all co-dominant	

MD: Marek's disease; SPF: specified pathogen free.

[a]Curator and institution are current to July 2006. Refer to http://animalscience.ucdavis.edu/AvianResources for contact information and also a larger listing of avian research resources, curators and contact information.

[b]Stock name, as designated by curator.

[c]Additional information on the stocks, as provided by the curators.

[d]Review and data articles as provided by curators for background on the stocks indicated; this is not an all inclusive listing.

TABLE A1.2 Inbred Lines Useful for Immunological Research

Location (Curator)[a]	Stock Name[b]	Origin and History	Description/Notes[c]	References[d]
Danish Institute of Agricultural Sciences (DIAS) (Juul-Madsen, H.R.)	DIAS B12-2	White Leghorn (WL), intercrossed for line maintenance	20+ generations; MHC	Hála (1987)
	DIAS B130-130	Scandinavian White Cornish (WC)	20+ generations; MHC: B21-like	Simonsen (1987)
	DIAS B131-131	Scandinavian WC	20+ generations; MHC: B21-like	Simonsen (1987)
	DIAS B13-133	WL, GB1	20+ generations; MHC	Hála (1987)
	DIAS B14-34	WL, Hy-Line origin	20+ generations; MHC: H.B14A	Godin *et al.* (2003)
	DIAS B15-22	WL from Cornell K line	20+ generations; MHC	Cole and Hutt (1973)
	DIAS B19-111 Scandinavian	Scandinavian WL	20+ generations; MHC: B19	Juul-Madsen *et al.* (1993)
	DIAS B19-131 Scandinavian	Scandinavian WC	20+ generations; MHC: B19	Juul-Madsen *et al.* (1993)
	DIAS B-1921	American WL	20+ generations; MHC: H-B19	Hála (1987)
	DIAS B19-39	American WL	20+ generations; MHC: H-B19	Hála (1987)
	DIAS B201-201	Scandinavian, Rhode Island Red (RIR)	20+ generations; MHC: B21-like	Juul-Madsen *et al.* (1993)
	DIAS B21-21	WL, Hy-Line origin	20+ generations; MHC: H-B21	Hála (1987)
	DIAS B2-32	Scandinavian WL	20+ generations; MHC: B2	Simonsen *et al.* (1982)
	DIAS B4-4	WL, intercrossed for line maintenance	20+ generations; MHC: B4	Hála (1987)

(*Continued*)

TABLE A1.2 (Continued)

Location (Curator)[a]	Stock Name[b]	Origin and History	Description/Notes[c]	References[d]
	DIAS B6-36	WL, GB2	20+ generations; MHC: B6	Hála (1987)
	DIAS BW1-111	Red jungle fowl (RJF), Copenhagen Zoo	20+ generations; MHC: B21-like	Simonsen (1987)
	DIAS BW3-13	RJF Copenhagen Zoo	20+ generations; MHC: BW3	Crone and Simonsen (1987)
	DIAS BW4-14	RJF Copenhagen Zoo	20+ generations; MHC: BW4	Crone and Simonsen (1987)
	DIAS MBL High-10	Danish WC Stock	8 generations	Juul-Madsen *et al.* (2004)
	DIAS MBL Low-10	Danish WC Stock	8 generations	Juul-Madsen *et al.* (2004)
	DIAS BR2	Recombinant WL	20+ generations; MHC, formerly named R2-1,	Briles *et al.* (1982), Simonsen *et al.* (1982)
	DIAS BR4	Hy-Line origin WL	20+ generations; MHC, formerly named R4-1	Briles *et al.* (1982), Koch *et al.* (1983)
	DIAS BR	Hy-Line origin WL	20+ generations; MHC, formerly named R5-1	Briles *et al.* (1992), Koch *et al.* (1983)
Institute for Animal Health (IAH) (Head of Animal Services)	IAH 6_1	WL ADOL 1972	MHC: B2; susceptible to ALV (avian leukosis virus) A, B, C and D. Resistant to ALV tumour development and Marek's disease (MD); expresses chB6a on B cells, histocompatible with IAH 7_2; inbreeding coefficient $F = 0.99$	Lee *et al.* (1981), Burgess *et al.* (2001)
	IAH 7_2	WL ADOL 1972	MHC: B2; susceptible to LLV (lymphoid leukosis virus) A, B, D and E. Resistant to LLV tumour development, highly susceptible to MD. Expresses chB6b on B cells, histocompatible with IAH 6_1; $F = 0.99$	Lee *et al.* (1981), Burgess *et al.* (2001)
	IAH N	WL ADOL 1982, originated Cornell	MHC: B21; highly resistant to MD	Burgess *et al.* (2001)
	IAH P2a	WL Lelystad, 1996, originated Cornell	MHC: B19; highly susceptible to MD	Burgess *et al.* (2001)
	IAH 0	WL, imported from ADOL 1985	MHC: B21; free of endogenous ALV genes (*ev* loci) by DNA hybridization; susceptible to infection by ALV subgroups A, B, C and D	
	IAH C	WL, 1969 imported from Cambridge, originated Reaseheath Poultry Breeding Station (RPBS)	MHC: mixed B4 and B12, susceptible to ALV subgroups B, C and D; resistant to MDV; $F = 0.99$	
	IAH 15I	WL, 1962 imported ADOL	MHC: B15, susceptible to ALV A and C, segregating for B, D and E; moderately susceptible to MD	Burgess *et al.* (2001)
	IAH W	WL, 1962 imported from Wellcome Research Laboratories	MHC: originally four congenic lines: B14A, B14B, B14C, B14D; represent each combination of two alleles at both IgM and IgY constant regions	

(*Continued*)

TABLE A1.2 (Continued)

Location (Curator)[a]	Stock Name[b]	Origin and History	Description/Notes[c]	References[d]
Institute of Molecular Genetics (Plachý, J.)	Bli	Brown Leghorn (BL), Prague 1966	MHC: B129; ALV endogenous gs^+; inbreeding $F_x = 0.78+F25$	Hála and Plachý (1997), Plachý (2000), Plachý *et al.* (1992)
	CB	WL, RPBS 1932, Prague 1958	White, MHC: B12; ALV endogenous gs^- chf^- ev-17^+; inbreeding $F25 + 45$	Hála and Plachý (1997), Plachý (2000), Plachý *et al.* (1992)
	CC	WL, Prague 1963	White, MHC: B4; ALV endogenous ev-17^-; inbreeding $F25+6+39$	Hála and Plachý (1997), Plachý (2000), Plachý *et al.* (1992)
	H6	WL, Regional Poultry Research Laboratory (RPRL – now ADOL), Houghton Poultry Research Station (HPRS), Prague 1989	MHC: B2; inbreeding $F_x = 0.99+F14$	Hála and Plachý (1997), Plachý (2000), Plachý *et al.* (1992)
	L15B	WL, RPRL 1939, Prague 1977	MHC: B15; ALV endogenous gs^-; inbreeding $F_x = 0.95+F18$, G4 F23	Hála and Plachý (1997), Plachý (2000), Plachý *et al.* (1992)
	M	Prague 1956	Black Minorca, MHC: B21x; inbreeding $F48$	Hála and Plachý (1997), Plachý (2000), Plachý *et al.* (1992)
	WA	RPBS 1941, Prague 1962	Barred Leghorn (BRDL), MHC: B9; ALV endogenous gs^+; inbreeding $+F19+$ 19G20	Hála and Plachý (1997), Plachý (2000), Plachý *et al.* (1992)
Iowa State University (ISU) (Lamont, S.J.)	ISU 19-13	Single Comb Leghorn (SCL). Derived from crosses of 1920s ISU inbreds before 1935	Inbreeding coefficient $F > 0.99$; MHC: B13	Lamont *et al.* (1992), Plotsky *et al.* (1995), Zhou and Lamont (1999)
	ISU 19-15.1	SCL. Derived from crosses of 1920s ISU inbreds before 1935	$F > 0.99$; MHC: B15.1	Lamont *et al.* (1992), Plotsky *et al.* (1995), Zhou and Lamont (1999)
	ISU 8-15.1	SCL. Derived from crosses of 1920s ISU inbreds before 1935	$F > 0.99$; MHC: B15.1	Lamont *et al.* (1992), Plotsky *et al.* (1995), Zhou and Lamont (1999)
	ISU GH-1	SCWL. Derived from Ghostley Hatchery, Minnesota (MN) females X H&N males in 1954	$F > 0.99$; MHC: B1	Lamont *et al.* (1992), Plotsky *et al.* (1995), Zhou and Lamont (1999)
	ISU GH-13	SCWL. Derived from Ghostley Hatchery (MN) females X H&N males in 1954	$F > 0.99$; MHC: B13	Lamont *et al.* (1992), Plotsky *et al.* (1995), Zhou and Lamont (1999)
	ISU GH-15.1	SCWL. Derived from Ghostley Hatchery (MN) females X H&N males in 1954	$F > 0.99$; MHC: B15.1	Lamont *et al.* (1992), Plotsky *et al.* (1995), Zhou and Lamont (1999)
	ISU HN-12	SCWL. Derived from a pure Kimber line from H&N in 1954	$F > 0.99$; MHC: B12	Lamont *et al.* (1992), Plotsky *et al.* (1995), Zhou and Lamont (1999)
	ISU HN-15	SCWL. Derived from a pure Kimber line from H&N in 1954	$F > 0.99$; MHC: B15	Lamont *et al.* (1992), Plotsky *et al.* (1995), Zhou and Lamont (1999)

(*Continued*)

TABLE A1.2 (Continued)

Location (Curator)[a]	Stock Name[b]	Origin and History	Description/Notes[c]	References[d]
	ISU M15.2	Fayoumi. Imported from Egypt in 1954, congenic with line ISU M5.1	$F > 0.98$; MHC: B15.1, original stock thought to be resistant to lymphoid leukosis (LL)	Lamont *et al.* (1992), Plotsky *et al.* (1995), Zhou and Lamont (1999)
	ISU M5.1	Fayoumi. Imported from Egypt in 1954, congenic with line ISU M15.2	Inbreeding coefficient $F > 0.98$; MHC: B5.1, original stock thought to be resistant to LL	Lamont *et al.* (1992), Plotsky *et al.* (1995), Zhou and Lamont (1999)
	ISU Sp21.2	Spanish chicken. Imported from Spain in 1954	$F > 0.99$; MHC: B21.2	Lamont *et al.* (1992), Plotsky *et al.* (1995), Zhou and Lamont (1999)
University of California, Davis (UCD) (Delany, M.E.)	UCD 001	RJF. Original stock from Malaysia, to Hawaii, to Cornell, to UC Berkeley (1940), to UCD	MHC: BQ (B21-like), $F > 0.80$, wild-type jungle fowl (phenotype: seasonal breeder, brown eggs, appropriate body size and plumage, red ear lobes). Parent line used for the East Lansing reference mapping population and genome sequencing	Abplanalp (1992), Crittenden *et al.* (1993), Delany and Pisenti (1998), ICGSC (2004)
	UCD 003	SCWL. Full-sib crosses since 1956,	MHC: B17, $F > 0.99$; A4/E7, C2 P3 blood cell alloantigens; resistant to Rous sarcoma (RS) and susceptible to MD. Serves as background line for UCD congenic lines (MHC and mutant); parent line used for the East Lansing reference mapping population	Abplanalp (1992), Crittenden *et al.* (1993), Delany and Pisenti (1998)
	UCD 077	SCWL. Developed in Switzerland, imported in 1987; full-sib inbreeding since 1968	MHC: B mixed: B15, B16, $F > 0.95$; high blood lipid levels; multifactorial	Abplanalp (1992)
University of New Hampshire (Smith, S.C.)	Show Racer Pigeons	Palmetto Pigeon Plant, Sumter, South Carolina (SC) 1962	Inbred pair mating, 50 pairs in closed flock since1975. Resistant to spontaneous (non-induced) atherosclerosis; single gene autosomal dominant	Smith and Taylor (2001)
	White Carneau Pigeons	Palmetto Pigeon Plant, Sumter, SC 1962	Inbred pair mating, 100 pairs in closed flock since 1975. Susceptible to spontaneous (non-induced) atherosclerosis; single gene autosomal recessive	Smith and Taylor (2001)
USDA-Avian Disease and Oncology Laboratory (ADOL) (Cheng, H.H)	ADOL 6_3	SCWL. Initiated in 1939	$F > 0.99$, MHC: B2; endogenous ALV ev1 and ev3; resistant to ALV and to MD; specific pathogen free	Bacon *et al.* (2000), Fulton *et al.* (1996), Stone (1975)
	ADOL 7_1	SCWL. Initiated in 1939	$F > 0.99$, MHC: B2; endogenous ALV not defined; susceptible to MD; specific pathogen free	Bacon *et al.* (2000), Fulton *et al.* (1996), Stone (1975)
	ADOL 7_2	SCWL. Initiated in 1939	$F > 0.99$, MHC: B2; endogenous viruses ev1 and ev2; resistant to ALV-A, -B and -E and susceptible to MD; specific pathogen free	Bacon *et al.* (2000), Fulton *et al.* (1996), Stone (1975)

(*Continued*)

TABLE A1.2 (Continued)

Location (Curator)[a]	Stock Name[b]	Origin and History	Description/Notes[c]	References[d]
	ADOL $15B_1$	SCWL. Initiated in 1939	$F > 0.95$; MHC: B5 and B15; no endogenous ALV; susceptible to all ALV; specific pathogen free	Bacon *et al.* (2000)
	ADOL Reaseheath Line C	SCWL. Initiated in 1932; highly inbred	MHC: B12; endogenous viruses ev1, ev7 and ev10; susceptible to ALV-B and -C, but resistant to ALV-A and ALV-E; specific pathogen free	Bacon *et al.* (2000)
	ADOL $15I_5$	SCWL. Initiated in 1939	$F > 0.99$, MHC: B15, endogenous ALV ev1, ev6, and ev10 or ev11; susceptible to ALV-A, -B and -E, and to MD; specific pathogen free	Bacon *et al.* (2000), Fulton *et al.* (1996), Stone (1975), Waters (1945)
	ADOL 6C.7	SCWL: 12% ADOL 7_2, 88% ADOL 6_3 in 19 different recombinant strains (RCS)	Each RCS has ~12.5% line 7_2 genes in a 6_3 background. Some RCS differ for plasma IgG and some for susceptibility to MD	Bacon *et al.* (2000)

[a]Curator and institution are current to July 2006. Refer to http://animalscience.ucdavis.edu/AvianResources for contact information and also a larger listing of avian research resources, curators and contact information.
[b]Stock name, as designated by curator.
[c]Additional information on the stocks, as provided by the curators.
[d]Review and data articles as provided by curators for background on the stocks indicated; this is not an all inclusive listing.

TABLE A1.3 Congenic Lines Useful for Immunological Research

Location (Curator)[a]	Stock Name[b]	Origin and History	Description/Notes[c]	References[d]
USDA-Avian Disease and Oncology Laboratory (ADOL) (Cheng, H.H)	ADOL 100B	SCWL. Initiated in 1962; congenic with ADOL 7_2	MHC: B2, endogenous viruses ev1 and ev2; resistant to ALV-A, -B, and -E; specific pathogen free	Stone (1975)
	ADOL 15.6-2	SCWL. Congenic with ADOL $15I_5$; initiated in 1979	MHC: B2 from ADOL 6-1; specific pathogen free	Briles *et al.* (1982), Fulton *et al.* (1996)
	ADOL 15.7-2	SCWL. Congenic with ADOL $15I_5$; initiated in 1979	MHC: B2 from ADOL 7-2; specific pathogen free	Briles *et al.* (1982), Fulton *et al.* (1996)
	ADOL 15.15I-5	SCWL. Congenic with ADOL $15I_5$; initiated in 1979	MHC: B5 from ADOL $15I_4$; specific pathogen free	Briles *et al.* (1982), Fulton *et al.* (1996)
	ADOL 15.C-12	SCWL. Congenic with ADOL $15I_5$; initiated in 1979	MHC: B12 from Reaseheath line C; specific pathogen free	Briles *et al.* (1982), Fulton *et al.* (1996)
	ADOL 15.P-13	SCWL. Congenic with ADOL $15I_5$; initiated in 1979	MHC: B13 from Cornell JM-P; specific pathogen free	Briles *et al.* (1982), Fulton *et al.* (1996)
	ADOL 15.N-21	SCWL. Congenic with ADOL $15I_5$; initiated in 1979	MHC: B21 from Cornell JM-N; specific pathogen free	Briles *et al.* (1982), Fulton *et al.* (1996)
	ADOL 15.P-19	SCWL Congenic with ADOL $15I_5$; initiated in 1979	MHC: B19 from Cornell JM-P; specific pathogen free	Briles *et al.* (1982), Fulton *et al.* (1996)

(*Continued*)

TABLE A1.3 (Continued)

Location (Curator)[a]	Stock Name[b]	Origin and History	Description/Notes[c]	References[d]
Institute of Molecular Genetics (Plachý, J.)	CB.15	WL, Prague 1985	White, MHC: B15; inbreeding N9 F9	Hála and Plachý (1997), Plachý (2000), Plachý *et al.* (1992)
	CB.7	WL, Prague 1967	White, MHC: B7; inbreeding N9 F7 N4 F16	Hála and Plachý (1997), Plachý (2000), Plachý *et al.* (1992)
	CB.9	WL, Prague 1985	White, MHC: B9; inbreeding N9 F9	Hála and Plachý (1997), Plachý (2000), Plachý *et al.* (1992)
	CB.ev17	WL, Prague 1993	White, MHC: B12; inbreeding N2 F7	Hála and Plachý (1997), Plachý (2000), Plachý *et al.* (1992)
	CB.R1	WL, Prague 1975	White, MHC: B12r1(F12G4); inbreeding N6 F20	Hála and Plachý (1997), Plachý (2000), Plachý *et al.* (1992)
	CB.R4	WL, Prague 1986	White, MHC: B21r3(F21G15); inbreeding N9 F8	Hála and Plachý (1997), Plachý (2000), Plachý *et al.* (1992)
	CB.R5	WL, Prague 1989	White, MHC: B12/B15r1(F15 G21); inbreeding N10 F4	Hála and Plachý (1997), Plachý (2000), Plachý *et al.* (1992)
	CC.13	WL, Prague 1983	White, MHC: B13; inbreeding N9 F11	Hála and Plachý (1997), Plachý (2000), Plachý *et al.* (1992)
	CC.21	WL, Prague 1986	White, MHC: B21x; inbreeding N9 F8	Hála and Plachý (1997), Plachý (2000), Plachý *et al.* (1992)
	CC.21-I	WL, Prague 1997	White, MHC: B21x; inbreeding N2 F5	Hála and Plachý (1997), Plachý (2000), Plachý *et al.* (1992)
	CC.ev17+	WL, Prague 1993	White, MHC: B4; inbreeding N2 F7	Hála and Plachý (1997), Plachý (2000), Plachý *et al.* (1992)
	CC.R1	WL, Prague 1977	White, MHC: B4r1(F4G12); inbreeding N6 F20	Hála and Plachý (1997), Plachý (2000), Plachý *et al.* (1992)
	CC.R2	WL, Prague 1978	White, MHC: B4r2(F4G7); inbreeding N4 F22	Hála and Plachý (1997), Plachý (2000), Plachý *et al.* (1992)
	CC.R4	WL, Prague 1986	White, MHC: B21r3(F21G15); inbreeding N5 + N4 F7	Hála and Plachý (1997), Plachý (2000), Plachý *et al.* (1992)
Iowa State University (ISU) (Lamont, S.J.)	ISU G.S1.19H	SCWL. Congenic with ISU G lines (G-B1, G-B2, GH-1, GH-13, GH-15.1)	MHC derived from S1 line	Lamont *et al.* (1992), Plotsky *et al.* (1995), Zhou and Lamont (1999)
	ISU G.S1.19L	SCWL. Congenic with ISU G lines (G-B1, G-B2, GH-1, GH-13, GH-15.1)	MHC derived from S1 line	Lamont *et al.* (1992), Plotsky *et al.* (1995), Zhou and Lamont (1999)
	ISU G.S1.1H	SCWL. Congenic with ISU G lines (G-B1, G-B2, GH-1, GH-13, GH-15.1)	MHC derived from S1 line	Lamont *et al.* (1992), Plotsky *et al.* (1995), Zhou and Lamont (1999)

(*Continued*)

TABLE A1.3 (Continued)

Location (Curator)[a]	Stock Name[b]	Origin and History	Description/Notes[c]	References[d]
	ISU G.S1.1L	SCWL. Congenic with ISU G lines (G-B1, G-B2, GH-1, GH-13, GH-15.1)	MHC derived from S1 line	Lamont *et al*. (1992), Plotsky *et al*. (1995), Zhou and Lamont (1999)
	ISU G-B1	SCWL. Congenic with ISU G-B2	$F > 0.99$; MHC: B13	Lamont *et al*. (1992), Plotsky *et al*. (1995), Zhou and Lamont (1999)
	ISU G-B2	SCWL. Congenic with ISU G-B1	$F > 0.99$; MHC: B6	Lamont *et al*. (1992), Plotsky *et al*. (1995), Zhou and Lamont (1999)
North Carolina State University (Ashwell, C.)	UNH 6.15-5	SCWL. Congenic with UNH 6-1 (transferred from University of New Hampshire 2007)	$F > 0.99$; MHC: B5	
	UNH 6.6-2	SCWL. Congenic with UNH 6-1 (transferred from University of New Hampshire 2007)	$F > 0.99$; MHC: B2	
	UNH-UCD 003.R2	SCWL. Congenic with UCD 003 (transferred from University of New Hampshire 2007)	MHC: BF2-BL2-BG23, B complex recombinant, backcrossed eight times to UCD-003	
	UNH-UCD 003.R4	SCWL. Congenic with UCD 003 (transferred from University of New Hampshire 2007)	MHC: BF2-BL2-BG23, B complex recombinant, backcrossed eight times to UCD-003	
University of California, Davis (Delany, M.E.)	UCD 253	SCWL. Congenic with UCD 003, Kimber derived B-system	MHC: B18, resistant to RS and MD	Abplanalp (1992)
	UCD 254	SCWL. Congenic with UCD 003, Hy-Line derived B-system	MHC: B15, susceptible to RS and MD	Abplanalp (1992)
	UCD 312	SCWL. Congenic with UCD 003; MHC from New Hampshire breed	MHC: B24, susceptible to RS and MD	Abplanalp (1992)
	UCD 330	SCWL. Congenic with UCD 003; MHC from Australorp inbred line, Hy-Line derived B-system	MHC: B21, resistant to RS and MD	Abplanalp (1992)
	UCD 331	SCWL. Congenic with UCD 003; MHC from a dwarf SCWL	MHC: B2, resistant to RS and MD	Abplanalp (1992)
	UCD 335	SCWL. Congenic with UCD 003; MHC from a commercial (Hy-Line derived) Richardson Mt. Hope SCWL	MHC: B19, susceptible to RS and MD	Abplanalp (1992)
	UCD 336	SCWL. Congenic with UCD 003; MHC from RJF	MHC: BQ (similar to B21), resistant to RS and MD	Abplanalp (1992)

(*Continued*)

TABLE A1.3 (Continued)

Location (Curator)[a]	Stock Name[b]	Origin and History	Description/Notes[c]	References[d]
	UCD 342	SCWL. Congenic with UCD 003; MHC from a cross of Ceylon Jungle fowl and RJF	MHC: BC, susceptible to RS; sex ratio problem, excess males at hatch (2M:1F)	Abplanalp (1992)

ALV: avian leukosis virus; MD: Marek's disease; RJF: Red Jungle Fowl; RS: Rous sarcoma; WL: White Leghorn.

[a]Curator and institution are current to July 2006. Refer to http://animalscience.ucdavis.edu/AvianResources for contact information and also a larger listing of avian research resources, curators and contact information.

[b]Stock name, as designated by curator.

[c]Additional information on the stocks, as provided by the curators.

[d]Review and data articles as provided by curators for background on the stocks indicated; this is not an all inclusive listing.

TABLE A1.4 Randombred Lines Useful for Immunological Research

Location (Curator)[a]	Stock Name[b]	Origin and History	Description/Notes[c]	References[d]
Cornell University (Johnson, P.)	Cornell Special C	SCWL. Developed at Cornell in 1935 by R. Cole; C strain with Kimber males	Presently compared with K strain for adenocarcinoma incidence	Cole and Hutt (1973), Johnson and Giles (2006)
	Cornell C Specials (B-13)	SCWL. MHC matched to Cornell Obese; closed flock for over 30 years; derived from Cornell C strain	Control line for Cornell Obese; MHC B13; also can produce a persistent right Mullerian duct (often leading to the formation of two oviducts) in affected females	Cole and Hutt (1973), Johnson and Giles (2006)
North Carolina State University (NCSU) (Christensen, V.L.)	NCSU Ohio-RBC1	Large white turkey. Derived from Ohio-RBC1, kept as closed flock		
	NCSU Ohio-RBC2	Large white turkey. Derived from Ohio-RBC2, kept as closed flock		
Northern Illinois University (Briles, W.E.)	NIU Pheasants	From state game bird hatcheries in Wisconsin and Illinois in 1986	Ringed-neck pheasants; population segregating for a variety of MHC (B) haplotypes; gene pool	
University of Arkansas (Anthony, N.)	Arkansas Giant Jungle Fowl	Giant Jungle Fowl. From Southeast Asia in 1950, one male and five females, kept as closed flock	Naturally highly resistant to RSV; probably moderately inbred (foundation flock of six birds in 1950)	
	Arkansas Brown Line B101	BL. Acquired from Smyth at U. Massachusetts in 1996; subline of ancestral parent stock for Smyth Line	MHC-matched control/parental line for Arkansas Smyth line B101; allele e_b	
	Arkansas Light Brown Leghorn B101	LBL. Acquired from Smyth at U. Massachusetts in 1996	MHC-matched control for Arkansas Smyth line B101, does not develop delayed amelanogenesis (autoimmune vitiligo)	

BL: Brown Leghorn; RSV: Rous sarcoma virus.

[a]Curator and institution are current to July 2006. Refer to http://animalscience.ucdavis.edu/AvianResources for contact information and also a larger listing of avian research resources, curators and contact information.

[b]Stock name, as designated by curator.

[c]Additional information on the stocks, as provided by the curators.

[d]Review and data articles as provided by curators for background on the stocks indicated; this is not an all inclusive listing.

TABLE A1.5 Selected Lines Useful for Immunological Research

Location (Curator)[a]	Stock Name[b]	Origin and History	Description/Notes[c]	References[d]
Agriculture and Agri-Food Canada (Silversides, F.)	UBC-RES	Japanese quail. From NCSU in 1988	Resistant cholesterol-induced atherosclerosis	Hoekstra *et al.* (1998, 2003), Thomson *et al.* (2002)
	UBC-SUS	Japanese quail. From NCSU in 1988	Selected for susceptibility to cholesterol-induced atherosclerosis	Hoekstra *et al.* (1998, 2003), Thomson *et al.* (2002)
Cornell University (Austic, R.E.)	Cornell K strain	SCWL. Closed flock since 1954; pedigree bred until 1971 and selected for egg production, then randombred	MHC: B15; resistant to leukosis complex (MD) by selection after natural exposure to the virus; selected for high egg production, egg size, body weight and other economic egg traits; randombred since 1971	Cole and Hutt (1973)
Institute for Animal Science (Weigend, S.)	RSV RES Line Gr	WL. Derived from crossbred parents from long established American commercial breeding program (1965)	Resistant to RSV-A and B; segregating for B2, B13, B14, B19 and B21; allele Gr	
	RSV RES Line Mr	WL. Derived from commercial pure line WL (Cashmen) (1967)	Resistant to RSV-A and B; homozygous B2; allele Mr	
	RSV RES Line R	WL. Derived from Cornell K (1965)	Resistant to RSV-A and B; homozygous B15; allele Rr	
	RSV SUS Line Gs	WL. Derived from crossbred parents from long established American commercial breeding program (1965)	Susceptible to RSV-A and B; segregating for B2, B13, B14, B19 and B21; allele Gs	
	RSV SUS Line Ms	WL. Derived from commercial pure line WL (Cashmen) (1967)	Susceptible to RSV-A and B; homozygous B2; allele Ms	
	RSV SUS Line S	WL. Derived from Cornell K (1965)	Susceptible to RSV-A and B; homozygous B15; allele Rs	
Iowa State University (ISU) (Lamont, S.J.)	ISU S1-19H	SCWL. Derived from two inbred Hy-Line strains in 1964	$F > 0.50$; MHC: B19; Ir-GAT_{high} allele linked to MHC	Lamont *et al.* (1992), Plotsky *et al.* (1995), Zhou and Lamont (1999)
	ISU S1-19L	SCWL. Derived from two inbred Hy-Line strains in 1964	$F > 0.50$; MHC: B19; Ir-GAT_{low} allele linked to MHC	Lamont *et al.* (1992), Plotsky *et al.* (1995), Zhou and Lamont (1999)
	ISU S1-1H	SCWL. Derived from two inbred Hy-Line strains in 1964	$F > 0.50$; MHC: B1; Ir-GAT_{high} allele linked to MHC	Lamont *et al.* (1992), Plotsky *et al.* (1995), Zhou and Lamont (1999)
	ISU S1-1L	SCWL. Derived from two inbred Hy-Line strains in 1964	$F > 0.50$; MHC: B1; Ir-GAT_{low} allele linked to MHC	Lamont *et al.* (1992), Plotsky *et al.* (1995), Zhou and Lamont (1999)
North Carolina State University (Christensen, V.L.)	NCSU Ohio-F	Large white turkey. Derived from Ohio-F, kept as closed flock	Selected for increased body weight at 16 weeks	
The Hebrew University (Cahaner, A.)	Ab	CMM. Originated from ANAK80 (synthetic breed, dam line) in 1987	Selected for *E. coli* antibodies; three lines (HH, LL, Control)	

(*Continued*)

TABLE A1.5 (Continued)

Location (Curator)[a]	Stock Name[b]	Origin and History	Description/Notes[c]	References[d]
	AS	CMM. Originated from ANAK60 & 80 meat dam lines (1999)	Selected for incidence of ascites; two lines (AS-RES, AS-SUS)	
University of Arkansas (Anthony, N.)	Arkansas Progressor	Derived from SCWL, maintained at U. Arkansas since approximately 1945; thought to be highly inbred	Selected 20 generations for tumour growth (progression) following formation with exposure to RS. Also known as Arkansas Rous Sarcoma Progression	
	Arkansas Regressor	Derived from F-1 and F-2 of crosses of SCWL and Giant Jungle Fowl	Selected 20 generations for tumour regression following formation with exposure to RS. Also known as Arkansas Rous Sarcoma Regression	
University of Arkansas (Erf, G.)	Arkansas Smyth Line B101	BL. Acquired from Smyth at U. Massachusetts in 1996; aka DAM line (delayed amelanogenesis), synthetic	Selected for high expression of delayed amelanogenesis (autoimmune vitiligo: increasing amount of white feathers with each moult); also blindness and thyroiditis; amelanosis now seen >85% of offspring; e^+ (colour) and multifactorial; probably multifactorial with variable expressivity	
University of California, Davis (Delany, M.E.)	UCD-Cornell Trisomic	SCWL. Acquired from Cornell in 1995	Chromosomal variant, line-. bred Trisomy or tetrasomy of the MHC/NOR chromosomes, GGA16; such aneuploids can hatch and reach maturity, but tetrasomics are often small and have poor production characteristics	Bloom *et al.* (1987), Delany *et al.* (1987), Delany *et al.* (1988)
University of Georgia (Burke, W.H.)	Athens AR	SCWL. Derived from a cross of Athens AR2.5 and AR3.0 in 1997	Selected for resistance to aflatoxin	
	Athens AR3.0	Japanese quail	Selected for resistance to aflatoxin	
USDA-Avian Disease and Oncology Laboratory (ADOL) (Cheng, H.H.)	ADOL Line 0	SCWL. Initiated in 1979	MHC: B21, closed line selected for the absence of endogenous ALV-E proviral genes and for resistance to endogenous but susceptibility to exogenous ALV; specific pathogen free	Bacon *et al.* (2000)
	ADOL 0.44-VB*S1	SCWL. Initiated in 2000	MHC: B21, closed line selected for the absence of endogenous ALV-E proviral genes and for susceptibility to endogenous and all exogenous ALV; specific pathogen free	
	ADOL 0.ALV6	SCWL. Initiated in 1989; transgenic	Transgene is ALV-A envelope gene; transgenic progeny are resistant to ALV-A and ALV-E	Crittenden (1991)
	ADOL-Cornell N	SCWL. Derived from Cornell randombred stock (1965)	MHC: B21, resistant to MD strain JM; specific pathogen free	Cole (1968), Bacon *et al.* (2001)

(*Continued*)

TABLE A1.5 (Continued)

Location (Curator)[a]	Stock Name[b]	Origin and History	Description/Notes[c]	References[d]
	ADOL-Cornell P	SCWL. Derived from Cornell randombred stock (1965)	MHC: B19, susceptible to MD strain JM; specific pathogen free	Cole (1968), Bacon *et al.* (2001)
Virginia Polytechnic Institute (Siegel, P.)	Virginia Antibody Line-High	Derived from a Cornell randombred SCWL, starting 1977	Selected over 25 generations for high antibody response to sheep red blood cells	
	Virginia Antibody Line-Low	Derived from a Cornell randombred SCWL, starting 1977	Selected over 25 generations for low antibody response to sheep red blood cells	

ALV: avian leukosis virus; BL: Brown Leghorn; MD: Marek's disease; RSV: Rous sarcoma virus; WL: White Leghorn.

[a]Curator and institution are current to July 2006. Refer to http://animalscience.ucdavis.edu/AvianResources for contact information and also a larger listing of avian research resources, curators and contact information.

[b]Stock name, as designated by curator.

[c]Additional information on the stocks, as provided by the curators.

[d]Review and data articles as provided by curators for background on the stocks indicated; this is not an all inclusive listing.

TABLE A1.6 General Lines Useful for Immunological Research

Location (Curator)[a]	Stock Name[b]	Origin and History	Description/Notes[c]	References[d]
Institute of Molecular Genetics (Plachý, J.)	P	BL. All-Union Oncological Centre (AUOC), Prague 1986	Outbred; free of endogenous DNA sequences of ALV	Hála and Plachý (1997), Plachý (2000), Plachý *et al.* (1992)

ALV: avian leukosis virus; BL: Brown Leghorn.

[a]Curator and institution are current to July 2006. Refer to http://animalscience.ucdavis.edu/AvianResources for contact information and also a larger listing of avian research resources, curators and contact information.

[b]Stock name, as designated by curator.

[c]Additional information on the stocks, as provided by the curators.

[d]Review and data articles as provided by curators for background on the stocks indicated; this is not an all inclusive listing.

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APPENDIX 2

Resources Available for Studying Avian Immunology

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INTRODUCTION
REFERENCES

INTRODUCTION

As already pointed out by several authors, although the chicken has the best studied immune system of the birds, the development of reagents and research tools for studying avian immune responses still lags far behind the plethora of available tools for studying the murine and human systems or, for that matter, other mammalian agricultural species. Many research groups have developed their own reagents and generally make these available to colleagues in other laboratories. In particular, many monoclonal antibodies which recognize surface markers on chicken cells have been produced and there have been a number of meetings of the Avian CD Nomenclature Workshop (see Ratcliffe *et al.*, 1993). At the first meeting in 1991 in Monteal a standard nomenclature for avian leukocyte markers was agreed. Now, many monoclonal antibodies recognizing avian leukocyte antigens are available commercially. Two companies in particular, AbD Serotec (http://www.ab-direct.com/index) and Southern Biotech (http://www.ab-direct.com/index), market a wide range of the anti-chicken reagents. Some monoclonal antibodies can be purchased from laboratories. For instance the valuable reagents produced by the late Dr Suzan Jeurissen (some described in Chapter 2) can be purchased from Central Institute for Animal Disease Control, Lelystad, Netherlands (http://www.cidc-lelystad.wur.nl). For other reagents the reader will need to directly contact individual researchers, obtaining their details from publications. Another means of obtaining information about reagents that have been developed, but not necessarily described in publications, is through the use of the International Veterinary Immunology Research Network (to subscribe to this network e-mail listproc@lists.umass.edu).

Lists of reagents for studying the immune responses of avian agricultural species have already been provided in Chapter 21, including a list (Table 21.2) of monoclonal antibodies that recognize surface markers on chicken cells but also cross-react with other species. In this appendix Table A2.1 provides a list of monoclonal reagents that recognize avian cytokines, and some additional cell surface markers that have not yet been commercialized. Table A2.2 provides information on the availability of recombinant chicken cytokines. A number of colleagues have provided information on cell lines that they have found useful in various assay systems and which can be made available on request. This information is provided in Table A2.3.

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TABLE A2.1 Monoclonal Antibodies to Avian Cytokines and Some Cell Surface Markers Not Yet Commercialized

Antigen[a] Recognized	Name of mAb	Isotype	Generated[b]	Utility[c]	References[d]	Available from[e]
IFN-γ	1E12	IgG1	Gene gun	ELISA, neutralizing	Lambrecht *et al.* (2004)	belam@var.fgov.be
	1D12	IgG1	Gene gun	ELISA, neutralizing	Lambrecht *et al.* (2004)	
	3B7	IgG1	Gene gun	FACS		
	7C4	IgG1	Gene gun	FACS		
	YC1	IgG2b		ELISA, Western	Yun *et al.* (2000)	HLILLEHO@anri.barc.usda.gov
	YC2	IgG2b		ELISA, Western		
	YC3	IgM		ELISA, Western		
	YC4	IgG1		ELISA, Western		
	YC5	IgG2b		ELISA, Western		
	YC6	IgG1		ELISA, Western		
	YC7	IgM		ELISA, Western		
	YC8	IgG1		ELISA, Western		
	YC9	IgG1		ELISA, Western		
	YC10	IgG1				
	2C1	IgM		ELISA, Western		
	6A7	IgM		ELISA, Western		
	G3-1	IgM		ELISA, Western		
	80.9	IgG1	DNA/protein boost	ELISA	Lowenthal *et al.* (2000)	john.lowenthal@csiro.au
	85.6	IgG2A	DNA/protein boost	ELISA, neutralizing		
	9.1	IgG2B	DNA/protein boost	ELISA, Western		
	1.13	IgM	DNA/protein boost	ELISA, Western		
IL-2	chIL-2/1	IgG2b		ELISA		HLILLEHO@anri.barc.usda.gov
	chIL-2/2	IgG1		ELISA		
	chIL-2/4	IgG1		ELISA		
	chIL-2/5	IgG1		ELISA		
	chIL-2/7	IgG2b		ELISA		
	chIL-2/8	IgG1		ELISA		
	chIL-2/11	IgG2b		ELISA		
	chIL-2/12	IgG1		ELISA		
	chIL-2/16	IgG1		ELISA		
	chIL-2/18	IgG2b		ELISA		
	chIL-2/19	IgG1		ELISA	Miyamoto *et al.* (2001)	
	LHS1	IgG1	DNA/protein boost	ELISA, neutralizing, Western	Hilton *et al.* (2002a)	john.lowenthal@csiro.au
	4F12	IgG2a	DNA/protein boost	ELISA, neutralizing, Western		pete.kaiser@bbsrc.ac.uk; Serotec
	10E7	IgG2b	DNA/protein boost	ELISA, neutralizing, Western		
IL-4	ED4	IgM	DNA/protein boost	ELISA		pete.kaiser@bbsrc.ac.uk
	GG6	IgM	DNA/protein boost	ELISA		
	AE11	IgG1	DNA/protein boost	ELISA		
	CB4	IgG2a	DNA/protein boost	ELISA		

(*Continued*)

TABLE A2.1 (Continued)

Antigen[a] Recognized	Name of mAb	Isotype	Generated[b]	Utility[c]	References[d]	Available from[e]
	CF8	IgG2b	DNA/protein boost	ELISA		
	GH4	IgG2b	DNA/protein boost	ELISA		
IL-6	1.12.4	IgG1			Scott and Lillehoj (2006)	HLILLEHO@anri.barc.usda.gov
	1.15.4	IgG2a				
	1.20.7	IgG2a				
	1.22.2	IgG2a				
	1.24.5	IgG2a				
	1.26.4	IgG2b				
	1.28.7	IgG2a				
	1.32.5	IgG2a				
	117.6B	IgG1	DNA/protein boost	ELISA, Western	Hilton *et al.* (2002b)	andrew.bean@csiro.au
	GD10	IgG2a	DNA/protein boost	ELISA		pete.kaiser@bbsrc.ac.uk
	HE11	IgG2a	DNA/protein boost	ELISA		
	IG9	IgG2a	DNA/protein boost	ELISA		
IL-10	CG3	IgM	DNA/protein boost			pete.kaiser@bbsrc.ac.uk
IL-12β	AE5	IgM	DNA/protein boost			pete.kaiser@bbsrc.ac.uk
	AG6	IgG1	DNA/protein boost			
	BE1	IgM	DNA/protein boost			
	DC8	IgG1	DNA/protein boost			
	HC8	IgG1	DNA/protein boost			
IL-13	AE12	IgG2a	DNA/protein boost			pete.kaiser@bbsrc.ac.uk
	DA5	IgG2a	DNA/protein boost			
	HD7	IgG1	DNA/protein boost			
	IC8	IgM	DNA/protein boost			
IL-15	L1-18	IgG1		ELISA, Western	Min *et al.* (2002)	HLILLEHO@anri.barc.usda.gov
	M2-3	IgG1		ELISA, Western		
	M4-1	IgG2a		ELISA, Western, neutralizing		
	M4-2	IgG1		ELISA, Western		
	M4-3	IgG1		ELISA, Western		
	M4-5	IgG2a		ELISA, Western		
	M4-7	IgG2b		ELISA, Western, neutralizing		
	M4-12	IgG1		ELISA, Western		
	M5-6	IgG2b		ELISA, Western		
	M5-17	IgG1		ELISA, Western		

(*Continued*)

TABLE A2.1 (Continued)

Antigen[a] Recognized	Name of mAb	Isotype	Generated[b]	Utility[c]	References[d]	Available from[e]
IL-15Rα	2.19.1	IgG1		ELISA, Western	Li *et al.* (2002)	HLILLEHO@anri.barc.usda.gov
	5.3.1	IgG3		ELISA, Western, neutralizing		
	4.17.1	IgG3		ELISA, Western		
	1.5.1	IgG1		ELISA, Western		
	2.16.2	IgG1		ELISA, Western		
	3.12.1	IgG1		ELISA, Western		
	1.22.1	IgG1		ELISA, Western		
	2.9.1	IgG1		ELISA, Western, neutralizing		
	2.17.2	IgG2b		ELISA, Western		
	1.16.2	IgM		ELISA, Western		
	2.15.2	IgG2a		ELISA, Western		
	1.12.1	IgG1		ELISA, Western		
IL-22	JB4	IgG1	DNA/protein boost			pete.kaiser@bbsrc.ac.uk
	DC1	IgM	DNA/protein boost			
IFN-α	8A9	rat IgG1	Protein	ELISA, neutralizing		Kaspers@tiph.vetmed.uni-muenchen.de
	UMCh3-IFN-α	IgG1	Recombinant IFN-α	ELISA, Western		
Common γ chain receptor	gM1-11					HLILLEHO@anri.barc.usda.gov
TLR7	TLR7	IgM	DNA/protein boost	FACS, immunohistology		andrew.bean@csiro.au
Duck CD4	Du CD4-1	IgG1	293T cells transfected with CD4	FACS		Kaspers@tiph.vetmed.uni-muenchen.de
	Du CD4-2	IgG2a	293T cells transfected with CD4	FACS		
Duck CD8α	Du CD8-1	IgG2b	293T cells transfected with CD8a	FACS		Kaspers@tiph.vetmed.uni-muenchen.de
	Du CD8-2	IgG3	293T cells transfected with CD8a	FACS		
Duck Ig light chain	14A3	IgG1	Purified yolk IgY	ELISA, Western, FACS		Kaspers@tiph.vetmed.uni-muenchen.de
Duck IgY heavy chain IgY and ΔFcIgY	16C7	IgG1	Purified yolk IgY	ELISA, Western, FACS		Kaspers@tiph.vetmed.uni-muenchen.de
Duck IgA α-chain	Du IgA2		Purified bile IgA	ELISA, Western		Kaspers@tiph.vetmed.uni-muenchen.de

[a] Chicken unless otherwise stated.
[b] Method of immunization.
[c] Where demonstrated and the information provided.
[d] Where provided.
[e] Reagents are grouped under the appropriate contact e-mail.

TABLE A2.2 Availability of Recombinant Chicken Cytokines

Cytokine	Source	Bioassay	References	Available from
IFN-γ	*E. coli*, baculovirus, adenovirus	HD11 cell NO assay	Lambrecht *et al.* (1999)	belam@var.fgov.be; farau@var.fgov.be
IL-4	*E. coli*, COS, baculovirus	MHC-II expression on macrophages		belam@var.fgov.be
IL-2	*E. coli*	Proliferation, IFN-γ induction from T cells		
IL-1β, IL-2, IL-6, IL-15, IL-8, IL-16, IL-17, IL-18, IFN-α, IFN-γ, TGF-β4	Unspecified			HLILLEHO@anri.barc.usda.gov
IFN-α	*E. coli*	Virus protection		andrew.bean@csiro.au
IFN-β	*E. coli*	Virus protection		
IFN-γ	*E. coli*	HD11 cell NO assay	Digby and Lowenthal (1995)	
IL-2	*E. coli*	Proliferation of T cells	Hilton *et al.* (2002a)	
IL-6	*E. coli*	Proliferation of 7TD1 cells		
IL-12	*E. coli*	Proliferation of T cells		
IL-18	*E. coli*	Proliferation of T cells		
cMGF	*E. coli*	Proliferation of 7TD1 cells		
IFN-α	*E. coli*, 293 cells	Reporter assay	Schultz *et al.* (1995)	Kaspers@tiph.vetmed.uni-muenchen.de
IFN-β	*E. coli*, 293 cells	Reporter assay	Schwarz *et al.* (2004)	
IFN-γ	*E. coli*, 293 cells	HD11 cell NO assay	Weining *et al.* (1996)	
IL-1β	*E. coli*, 293 cells	Reporter assay	Gyorfy *et al.* (2003)	
IL-6	*E. coli*, 293 cells	Proliferation of 7TD1 cells	Schneider *et al.* (2001)	
IL-18	*E. coli*	2D8 cell based bioassay	Schneider *et al.* (2000)	
BAFF	*E. coli*, 293 cells	Survival of primary B cells (spleen)	Schneider *et al.* (2004)	
All 81 cytokines and chemokines so far identified in the chicken genome have been cloned, some with multiple isoforms. The majority are available as recombinants	Mainly from COS cells but some from *E. coli* and baculovirus	Bioactivity has not been demonstrated for all		pete.kaiser@bbsrc.ac.uk

BAFF: B cell activating factor.

Lack of suitable cell lines for avian immunological studies has been a major stumbling block that has hampered progress in the development of functional assays such as cell cytotoxicity and natural killer (NK) cell activity. This is despite the availability of large numbers of immortal chicken cell lines, most of them virally transformed. A nomenclature system for avian cell lines has been devised by a committee chaired by R.L. Witter (see Nazerian, 1979). Later Nazerian (1987) published lists of cell lines that were available at that time, and these still provide a very useful source of information, especially on the origins of some of the classical cell lines. One of the best examples is the LSCC-RP9 cell line, which has proved a valuable, although incompletely understood, target cell in chicken NK cell assays (see Sharma and Okazaki, 1981; Chapter 7). Thacker *et al.* (1995) demonstrated that this cell line can be

TABLE A2.3 Some Avian Cell Lines and Their Uses

Cell-Line Name	Transforming Agent	Species	Strain (MHC Type)	Cell Type	Use	References	Contact Details
2D8	REV-T	Chicken	WL B19/B19	B lymphocyte	IFN-γ secretion upon IL-18 stimulation	Puehler *et al.* (2003)	kaspers@tiph.vetmed.uni-muenchen.de
B19 REV-B	REV-T	Chicken	WL B19/B19	B cells from bursa of Fabricius	Antigen presenting cells	Haeri *et al.* (2005)	shayan@uoguelph.ca
B21 REV-B	REV-T	Chicken	WL B21/B21	B cells from bursa of Fabricius	Antigen presenting cells	Haeri *et al.* (2005)	shayan@uoguelph.ca
CEC32-Mx-LUC	Unknown	Quail	Unknown	Fibroblast	IFN-α/β assay	Schwarz *et al.* (2004)	peter.staeheli@uniklinik-freiburg.de
CEC32-NFkB-LUC	Unknown	Quail	Unknown	Fibroblast	IL-1β assay	Gyorfy *et al.* (2003)	peter.staeheli@uniklinik-freiburg.de

WL: White Leghorn.

transfected with major histocompatibility complex (MHC) class I genes from different inbred lines of chicken to generate MHC-specific immortal target cell lines for use in cytotoxic lymphocyte (CTL) assays. Levels of CTL killing in chromium release assays proved remarkably high in avian leukosis virus model infection, indicating these transfected cells could be very useful for CTL investigations with other viral diseases. By contrast, it has proved remarkably difficult to demonstrate cytotoxic killing using Marek's disease virus (MDV)-transformed cell lines as targets. Schat and co-workers developed a heterologous system for investigating cytotoxic responses in Marek's disease infection, based on targets that were reticuloendothelial virus-transformed cells stably transfected with specific MDV genes, and expressing their peptides (for review see Schat and Markowski-Grimsrud, 2001). In a series of experiments this heterologous system proved very useful for identifying the important MDV target proteins for CTL; however, the amount of CTL killing was very low compared to mammalian CTL assays.

The information provided in this appendix is by no means exhaustive. Thanks are expressed to those colleagues who responded to an e-mail enquiry sent out on the International Veterinary Immunology Research Network.

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GLOSSARY

AcLDL	Acetylated low-density lipoprotein
ACTH	Adrenocorticotropic hormone
ADCC	Antibody-dependent cell cytotoxicity
ADOL	Avian Disease and Oncology Laboratory
AECA	Anti-endothelial cell antibodies
AI	Avian influenza
AID	Activation-induced (cytidine) deaminase
AIV	Avian influenza virus
ALV	Avian leukosis virus
AMP	Antimicrobial peptides
aMPV	Avian metapneumovirus
ANA	Antinuclear antibodies
APC	Antigen-presenting cells
APP	Acute phase proteins
ARP	Avian respiratory macrophages or phagocytes
ART	Avian rhinotracheitis virus
ASC	Antibody-secreting cells
B-	Bursal-derived (cells)
BAC	Bacterial artificial chromosome
BAFF	B-cell activating factor
BALT	Bronchus-associated lymphoid tissue
BCR	B cell receptor
BL	Basal lamina
BL	Brown leghorn
BMR	Basal metabolic rate
BRDL	Barred leghorn
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
BSDC	Bursal secretory dendritic cells
CALT	Conjunctiva-associated lymphoid tissue
CAM	Chorioallantoic membrane
CBH	Cutaneous basophil hypersensitivity
cCAF	Chicken chemotactic and angiogenic factor
CD	Cluster of determination (suffixed by a number and sometimes Ligand: L)
CDK2	Cyclin-dependent kinase 2
CDR	Complementarity determining regions
CENP-A	Centromere protein A
CFA	Complete Freund's adjuvant
chBAFF	Chicken homologue BAFF
chCCL	Chicken CCL
chGG	Chicken gamma globulin
chIR	Chicken Ig-like receptor
chTR	Chicken telomerase RNA

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CIAV	Chicken infectious anaemia virus
CKC	Chick kidney cells
CMAFC	Cortico-medullary arch-forming cell
cMGF	Chicken myelomonocytic growth factor
CMI	Cell-mediated immune or cell-mediated immunity
CNPV	Canarypox virus
ConA	Concanavalin A
CRF	Corticotrophin-releasing factor
CP	Classical pathway
CpG	Cytosine–phosphate–guanosine (motif)
CRD	Carbohydrate-binding domains
CRP	C-reactive protein
CS	Cornell C strain
CSF	Colony-stimulating factors
CSS	Capsule of Schweigger-Seidel sheath
CT	Caecal tonsil
CtBP	Carboxyl-terminal-binding protein
CTL	Cytotoxic T lymphocytes
CTLA	CTL-associated antigen
DAM	Delayed amelanosis
DC	Dendritic cells
DES	Diethylstilboestrol
DH	Delayed hypersensitivity
DHBV	Duck hepatitis B virus
DHE	Dehydroepiandrosterone
DIAS	Danish Institute of Agricultural Sciences
DNA	Deoxyriboneucleic acid
DNP	Dinitrophenol
dsRNA	Double-stranded RNA
DTH	Delayed-type hypersensitivity
EARC	Ellipsoid-associated reticular cells
EBERs	EBV-encoded small RNAs
EBV	Epstein–Barr virus
ecm	Epithelium containing medullary
ECM	Extracellular matrix
EDS	Egg drop syndrome
efm	Epithelial-free medullary
EID	Embryonic incubation day
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot
eQTL	Expression-QTL
ERC	Epithelial reticular cells
ESC	Embryonic stem-cell
EST	Expressed sequence tag
FAE	Follicle-associated epithelium
FAE-SC	Follicle-associated epithelium supportive cell
FARM	Free avian respiratory macrophages
FB	Fibrinogen
FDC	Follicular dendritic cells
FITC	Fluorescein isothiocyanate

Fm	Fibromelanotic mutation
FPV	Fowlpox virus
GALT	Gut-associated lymphoid tissue
GC	Germinal centres
GFP	Green fluorescent protein
GM-CSF	Granulocyte/macrophage colony-stimulating factor
GVH	Graft-versus-host
GvHR	Graft-versus-host response
HA	Haemagglutinin
HBV	Hepatitis B virus
HC	Haematopoietic cells
HEV	High endothelial venules
HG	Harderian gland
HPA	Hypothalamic-pituitary-adrenal
HPRS	Houghton Poultry Research Station
HPV	Human papilloma viruses
HSC	Haematopoietic stem cells
HSP	Heat shock protein
HVT	Herpesvirus of turkey
IAH	Institute for Animal Health
IBDV	Infectious bursal disease virus
IBV	Infectious bronchitis virus
IC	Inositol cytosine (prefixed with poly)
ICP	Infected cell protein
IDC	Interdigitating dendritic cells
IEL	Intraepithelial leukocytes
IFE	Interfollicular epithelium
IFN	Interferon (suffixed by α, β or γ)
Ig	Immunoglobulin (may be suffixed by A, D, E, G, M or Y and may be prefixed by surface: s)
IgY(ΔFc)	Truncated form of IgY found in ducks
IH	Immediate hypersensitivity
IL	Interleukin (suffixed by a number)
IMCOP	Immunological correlate of protection
IML	Infiltrating mononuclear leukocytes
iNOS	Inducible nitric oxide synthase
ISU	Iowa State University
ITAM	Immunoreceptor tyrosine-based activation motifs
ITIM	Immunoreceptor tyrosine-based inhibition motifs
KIR	Killer Ig-like NK receptors
KLH	Keyhole limpet haemocyanin
LBL	Light brown leghorn
LD	Linkage disequilibrium
LE	Linkage equilibrium
LITAF	LPS-induced TNF-α factor
LL	Lymphoid leukosis
LLV	Lymphoid leukosis virus
LP	Lectin pathway

LPD	Lymphoproliferative disease
LPS	Lipopolysaccharide
LRC	Leukocyte receptor complex
LRR	Leucine-rich repeat
LT	Lymphotoxin
LTA	Lipoteichoic acid
LTR	Long terminal repeat
mAb	Monoclonal antibody
MAC	Membrane attack complex
MALT	Mucosa-associated lymphoid tissue
MAPK	Mitogen-activated protein kinase
MAS	Marker-assisted selection
MASP	MBL-associated serine proteases
MATSA	Marek's disease tumour-associated surface antigens
MBL	Mannan-binding lectin
MCP	Monocyte chemotactic protein
M-CSF	Macrophage colony-stimulating factor
MD	Marek's disease
MDA	Maternally-derived antibody
MDA-5	Melanoma differentiation-associated gene-5
MDV	Marek's disease virus
MEQ	*M*DV *Eco*RI-*Q*
MHC	Major histocompatibility complex
MIC	Methyl isocyanate
mIgM	Membrane-bound immunoglobulin M
MIP	Macrophage inflammatory protein
MIS	Multiple-head injector systems
MLN	Mural lymph nodes
MRC	Mesenchymal reticular cells
MW	Molecular weight
NAb	Natural antibodies
NALT	Nasal-associated lymphoid tissue
NCSU	North Carolina State University
NDV	Newcastle disease virus
NE	Necrotic enteritis
NF	Nuclear factor
NFκB	Nuclear factor-kappa B
NIU	Northern Illinois University
NK	Natural killer
NKC	NK complex
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
NOR	Nucleolar organizing region
NOS	Nitric oxide synthase
NRC	National Research Council
NT	Notochord
O/W	Oil-in-water
ODN	Oligodeoxynucleotide
ORF	Open reading frame
OS	Obese strain

PALS	Peri-arteriolar lymphatic sheath
PAMP	Pathogen-associated molecular patterns
PAP	Peroxidase anti-peroxidase
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood monocytic cells
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PGE_2	Prostaglandin E2
PHA	Phytohaemagglutinin
PHL	Poultry Health Laboratory
PIAS	Protein inhibitors of activated STAT
pIgR	Polymeric Ig receptor
PKR	Protein kinase receptor
PP	Peyer's patches
PrP	Prion protein
PRR	Pattern recognition receptors
PUFA	Polyunsaturated fatty acids
PWP	Peri-ellipsoidal white pulp
QTL	Quantitative trait loci
RAG	Recombination-activating gene
RCS	Recombinant strains
REV	Reticuloendotheliosis virus
RIG-I	Retinoic acid-inducible gene I
RIR	Rhode Island Red
RJF	Red jungle fowl
RMR	Resting metabolic rates
RNA	Ribonucleic acid (may be prefixed by ds, m or ss)
ROS	Reactive oxygen species
RPBS	Reaseheath Poultry Breeding Station
RPRL	Regional Poultry Research Laboratory
RS	Rous sarcoma
RSS	Recombination signal sequences
RSV	Rous sarcoma virus
RT-PCR	Reverse transcriptase polymerase chain reaction
SAT	Spontaneous autoimmune thryoiditis
SC	Secretory component
SCL	Single Comb Leghorn
SEREX	Serological expression cloning of tumour antigens
SL	Smyth line
SLE	Systemic lupus erythematosus
SLV	SL Vitiligo
SNP	Single-nucleotide polymorphism
SOCS	Suppressor of cytokine signalling
SPF	Specified pathogen-free
SPI2	*Salmonella* pathogenicity island 2
SRBC	Sheep red blood cells
SSc	systemic sclerosis
T-	Thymus-derived (cells)
TAP	Transporters for antigen processing

TCR	T cell receptor
αβ-TCR	Alpha-beta T cell receptor
γδ-TCR	Gamma-delta T cell receptor
TdT	Terminal deoxynucleotide transferase
Tg	Thyroglobulin
TGF	Transforming growth factor
Th	T helper 1 or 2
TIR	Toll interleukin 1-like receptor
TLR	Toll-like receptor
TM	Transmembrane
TNF	Tumour necrosis factor
TNFR	TNF receptor
TNFRSF	TNFR superfamily
TNFSF	TNF superfamily
TNP	Trinitrophenol
TPA	12-*O*-tetradecanoylphorbol-13-acetate
TRAF	TNFR-associated factor
Treg	T regulatory
TRIM	Tripartite motif
TRP	Tyrosinase-related protein
TRT	Turkey rhinotracheitis
TSH	Thyroid stimulating hormone
TSLP	Thymic stromal lymphopoietin
TTRAP	TRAF6/TNFR-associated protein
UCD	University of California, Davis
UNH	University of New Hampshire
VIP	Vasoactive intestinal peptide
VN	Virus-neutralizing
vTR	Virus-encoded RNA telomerase
vv	Very virulent
vvMDV	Very virulent MD virus
W/O	Water-in-oil
WL	White Leghorn

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