Order Number 9223924

The role of bronchus-associated lymphoid tissue in respiratory immunity of chickens and turkeys: Morphologic and functional studies

Fagerland, Jane Ann, Ph.D.

Iowa State University, 1992

U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

The role of bronchus-associated lymphoid tissue in respiratory immunity of chickens and turkeys: morphologic and functional studies

by

Jane Ann Fagerland

A Dissertation Submitted to the

Graduate Faculty in Partial Fulfillment of the

Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Department: Microbiology, Immunology, and Preventive Medicine
Major: Immunobiology

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

For the Major Program

Signature was redacted for privacy.

For the Major Department

Signature was redacted for privacy.

For the Graduate College

Iowa State University
Ames, Iowa

1992

To my daughter Erika

You make it all worthwhile

TABLE OF CONTENTS

PART II. STRUCTURE AND DEVELOPMENT OF BRONCHUS-ASSOCIATED LYMPHOID TISSUE IN CHICKENS

PART III. QUANTITATION AND DISTRIBUTION OF PLASMA CELLS, T LYMPHOCYTE SUBSETS, AND B LYMPHOCYTES IN BRONCHUS-ASSOCIATED LYMPHOID TISSUE OF CHICKENS: AGE-RELATED DIFFERENCES

GENERAL INTRODUCTION

Respiratory disease in poultry remains a stubborn problem resulting in large losses to poultry producers, despite the widespread availability of vaccines (68). A lack of basic information about the mechanisms which bring about protection in the respiratory tract has prevented the rational development of effective vaccines and other preventative measures (33).

In mammals, lymph nodes draining the respiratory tract are key tissues involved in generating antibody responses to antigens entering the lungs (18,76). Lymph nodes provide a microenvironment in which all the elements needed to initiate an immune response can interact efficiently (174). These elements, which are the same in birds and mammals, include properly processed antigen, antigen-presenting cells (macrophages, B lymphocytes, and dendritic cells), and T lymphocytes (166). Birds lack a system of lymph nodes comparable to that found in mammals; however, in birds there are focal nodules of lymphoid tissue in most organs of the body, including the lung (50). Because they include germinal centers, these parenchymal lymphoid nodules in birds also contain all of the elements needed to generate immune responses and may functionally compensate for the lack of lymph nodes.

In the primary bronchus of the avian lung, there are prominent lymphoid nodules at points where secondary bronchi open into the major airway (23). These form the bronchus-associated lymphoid tissues (BALT). The structure of

avian BALT is similar to that of other mucosa-associated lymphoid tissues (MALT), a family of tissues located at the interfaces of epithelial surfaces and the external environment. Certain morphologic features are common to MALT, regardless of tissue or species origin. MALT are composed of a population of lymphocytes covered by an epithelium which is structurally different from that covering adjacent non-lymphoid regions. The structural differences include features which facilitate uptake of foreign materials from the environment, including attenuation of apical cytoplasm, large numbers of apical vesicles and tubules, effaced microvilli, and an altered glycocalyx (184).

In gut-associated lymphoid tissue (GALT), there is evidence that antigens processed in GALT stimulate the preferential differentiation of IgA plasma cells. Since IgA is the predominant intestinal antibody in many species and has special properties making it especially effective at protecting mucosal surfaces, a model for a mucosal immune system has been proposed. In this model, interlinked MALT are responsible for generating mucosal immune responses (primarily secretory IgA in type) that are strictly independent of the systemic immune responses mediated by lymph nodes and spleen (reviewed in 144). While this model explains some types of mucosal immune responses, particularly in the gastrointestinal and upper respiratory tracts (17,116,176), there are enough examples in the literature of unexpected responses to mucosally presented antigens to question its universal applicability to all mucosal tissues in all species (62,92,114,151,158,177). The fact that

combinations of parenteral and mucosal routes of immunization often provide the best mucosal immune responses (88,125,171) suggests that mucosal and systemic immune responses overlap, each making important contributions that synergize to protect tissues from mucosal pathogens.

Objectives of Dissertation Research

Observations of increased numbers of BALT nodules in turkeys infected with *Bordetella avium* suggest that BALT may play a role in the immune response against this agent (167). The objective of this research was to study the role of BALT in respiratory protection in turkeys and chickens using morphologic and functional studies. The specific objectives were to 1) characterize the structure of BALT in chickens and turkeys and to determine if its structure changes during development; 2) to characterize the lymphocyte and plasma cell populations in chicken BALT and to note any developmental differences; and 3) to determine if macromolecular and particulate materials are able to cross the BALT epithelium in turkeys; and if so, whether this transport occurs preferentially in BALT follicle-associated epithelium (FAE) compared to non-FAE.

Explanation of Dissertation Format

This dissertation is presented in the alternate format and consists of four manuscripts. The first manuscript has been published in the *American Journal*

of Anatomy. The second will be submitted to *Avian Diseases* as a short research note. The third and fourth manuscripts will be submitted as original research reports to *Regional Immunology*, a journal devoted to studies of lymphoid tissues and organs. The format of each manuscript is of the journal in which it is intended for publication. The Review of Literature precedes the first manuscript, and a General Summary follows the last manuscript. References cited in sections other than the four manuscripts are in the Additional Literature Cited section following the General Summary.

The Ph.D. candidate was the principal investigator for each study.

LITERATURE REVIEW

Respiratory Disease in Poultry

Respiratory disease is a significant and persistent economic problem in the poultry industry. Each year respiratory disease results in indirect losses due to carcass condemnations, decreased egg production, and poor feed efficiency, costing producers hundreds of millions of dollars (69). These losses are compounded by direct costs of vaccination and treatment of respiratory problems.

A variety of pathogenic bacteria, viruses, and fungi are responsible for respiratory diseases in poultry. Infections with *Bordetella avium* (9), *Chlamydia psittaci* (67), *Aspergillus flavus,* and *Aspergillus fumigatus* (40) can cause significant disease losses in turkey flocks, while major problems in chicken flocks are due to infection with infectious bronchitis virus (89), laryngotracheitis virus (73), Newcastle disease virus (NDV) (3), and *Hemophilus paragallinarum* (187). Avian influenza virus (49), *Escherichia coli* (69), *Pasteurella multocida* (137), *Mycoplasma gallisepticum*, and *M. synoviae* (188) cause economically important respiratory disease in both chickens and turkeys.

The above-mentioned organisms are primary pathogens capable of causing disease alone; however, field outbreaks of poultry respiratory disease usually involve several agents. Infection with one organism may predispose birds to secondary infections (68); and although a primary infection may cause

only mild respiratory disease, infection with a second organism may dramatically increase mortality (146).

Respiratory disease may complicate other aspects of poultry operation as well. For example, adverse reactions or hyporesponsiveness to vaccination for unrelated diseases often occur when birds suffering from relatively mild respiratory disease are vaccinated (9).

Vaccines for many avian respiratory diseases are available, but these are often of limited usefulness for a variety of reasons. Commercially available live or modified-live vaccines effective against several viral respiratory pathogens and *P. multocida* provide good protection; however, control of vaccine strain virulence in these vaccines can be a serious problem. For example, new viral strains of augmented virulence may arise and be selected for in birds vaccinated for infectious bronchitis, avian influenza and infectious laryngotracheitis (70,77,79). Live vaccines against *P. multocida* often result in death losses of from 2 - 4% in turkey flocks (137).

Antigenic differences between strains of the same pathogen have prevented development of effective vaccines for *E.coli*, chlamydia, infectious bronchitis virus, and avian influenza virus. Lack of information about virulence factors and the host immune response to *B. avium* has stymied effective vaccine development against this pathogen.

Rational development of protective strategies against respiratory pathogens requires basic knowledge about both the pathogen and the host's

reaction to the pathogen. Different types of immune responses effect protection against different avian respiratory pathogens, depending upon *pathogen-centered* considerations such as virulence factors. Thus, an ideal vaccine (i.e., one that can easily and economically be administered to large flocks of birds and will induce long-lasting protection in the respiratory tract) will have to be custom-designed for each pathogen to some degree. However, current attempts to develop vaccines are frustrated by a lack of knowledge of *host-centered* considerations, such as basic information about the tissue sites and cells involved in generating protective respiratory immune responses in the avian respiratory tract.

Non-Specific Avian Respiratory Defense Mechanisms

Although there have been some studies of respiratory defense mechanisms in birds, much information has been extrapolated from work done in mammals. In many cases, birds and mammals share the same respiratory defense mechanisms. There are, however, important differences between the two groups of animals in effector cell types and perhaps in the mechanisms of protection, as well.

Aerodynamic filtration

Repeated branching of airways in the avian respiratory tract provides for removal of progressively smaller particles from the air stream via inertial

impaction, interception, and diffusion-capture (74). Purification of the airstream in this way has been termed *aerodynamic filtration*. Large particles (from 3.7 to 7 μm in diameter) are deposited in the nasal cavity and upper trachea, while mid-size particles (1.1 μm) are intercepted in the lung and posterior air sacs. The smallest particles (<1.1 μm) escape capture until they reach the posterior air sacs. Particles trapped by aerodynamic filtration are ultimately removed from the lung by mucociliary clearance and phagocytes.

Mucociliary clearance

Mucociliary clearance is a second important non-specific protective mechanism which clears foreign materials from avian lungs and airways. Particulate matter trapped in mucus sheets is swept cranially by the coordinated motion of cilia on tracheal and bronchial epithelial cells, then either swallowed or expelled to the outside by coughing and sneezing. Breakdown of this mechanism occurs in diseases characterized by loss of ciliated cells (such as bordetellosis and Newcastle disease) and may predispose birds to secondary infections, or even contribute to clinical symptoms of respiratory disease if mucus accumulates in airways (63).

Soluble factors

Soluble factors are important mediators of respiratory defense in mammals and are probably important in birds, as well. These factors include

microbicidal agents such as lysozyme, interferon, lactoferrin, peroxidase, proteases, and complement (109,183). In addition to serum- and phagocyte-derived agents, respiratory epithelial cells are also capable of producing antimicrobial substances in mammals (45). Information concerning analogous factors in the avian respiratory tract is very limited.

In supernatants of cultured chicken lymphocytes and macrophages, an interferon-like activity which prevents cytopathic effects of vesicular stomatitis virus in chick embryo fibroblast cultures (132,178) and activates chicken macrophages has been described (178). Proteins of three different molecular weights (17, 20 and 36 kilodaltons) have been purified from these types of cell cultures, but have not been fully characterized (57,132). The degree of similarity to mammalian interferon thus is not known.

There are few reports of interferon activity in the avian respiratory tract. In one of these, interferon-like activity was recovered from tracheal swabs in chickens infected with infectious bronchitis virus (118).

Cell-Mediated Avian Respiratory Defense Mechanisms

Cell-mediated defense is carried out by a wide variety of cells: cytotoxic T lymphocytes (CTL), macrophages, natural killer (NK) cells, and granulocytes. Macrophages, CTL, and granulocytes are distinct cell populations with specific phenotypes that can be identified by the presence of marker enzymes and

surface molecules. However, the NK cell in birds is a functional designation and several cell types are currently associated with NK killing activities.

Phagocytic cells

In mammals, pulmonary alveolar macrophages provide a major first line of defense against foreign materials and microorganisms that have evaded aerodynamic filtration and the mucociliary apparatus (28). Large numbers of alveolar macrophages (3-15 x 10^6 cells/gram of lung) can readily be obtained by bronchial lavage of unstimulated mammalian lungs. Similar techniques applied to unstimulated turkey and chicken lungs yield at least ten-fold fewer cells (in the range of 2-3 x 10^5 cells/bird), even when differences in body weights are standardized between birds and mammals (54,164).

Early studies regarded the cells washed from avian lungs to be macrophages (164, 653), but later studies identified the majority of the cells as heterophils (115), the avian homologue of the mammalian neutrophil. In a definitive morphologic study using light and electron microscopy, the three most common leukocytes in lung lavage fluid from chickens were found to be heterophils, lymphocytes, and macrophages, in descending order of prevalence (58).

It has been suggested that the small number of macrophages in avian lungs, as well as the lack of myeloperoxidase and catalase in avian heterophils, explains the susceptibility of poultry to respiratory disease (162). However,

avian heterophils and extracts of their granules are microbicidal for *E. coli*, *Staphylococcus albus*, *Serratia marcescens*, and *Candida albicans* (30,31). This killing has been attributed to lysozyme and cationic proteins in heterophil granules.

Avian heterophils provide some degree of protection against respiratory pathogens *in vivo* (162,163,165). Chickens in which heterophils were non-specifically attracted into the lung with *P. multocida* were shown to be protected against subsequent challenge with a virulent strain of *E. coli* (165). Protected birds had reduced mortality and morbidity, less severe gross and histological lesions, and greater weight gains at 3 days post-challenge than did controls (165). It seems, then, that heterophils may be able to functionally compensate for the lack of pulmonary macrophages in avian lungs.

In addition to lung heterophils, epithelial cells of gas-exchange regions in avian lung phagocytose foreign materials (154). In ducks exposed to iron oxide aerosols, particles were found in epithelial cells of the atria and infundibula, as well as in interstitial macrophages (155). Thus, in avian species, foreign particles which escape aerodynamic filtration, mucociliary clearance, and phagocytosis by heterophils may still be removed by phagocytic epithelial cells and macrophages within deep lung tissues.

Spontaneous cell-mediated cytotoxicity

Spontaneous cell-mediated cytotoxicity is a surveillance activity carried out by NK cells in the absence of previous sensitization to foreign antigens. In mammals, NK cells in the lungs are important defenders against viral infection, particularly early in infection before specific immunity has developed (156). The role of NK cells in avian respiratory defense has not been explored. However, studies have documented avian NK cell activity against intestinal coccidia (93), tumor cells, and non-respiratory viruses (i.e., Marek's disease and infectious bursal disease viruses) (130).

NK cells in birds have many of the same characteristics as mammalian NK cells and may have similar functions as well. Both mammalian and avian NK cells lack reactivity with monoclonal antibodies specific for macrophages and T and B lymphocytes (147), express asialoGM1 on their surfaces (147), and have large granular lymphocyte and lymphokine-activated killer homologs (147). NK cells from both species carry out their cytotoxic activities independently of major histocompatibility restriction and recognize neither virus- nor tumor-specific antigens on target cell surfaces (52). Avian cells shown to have NK activity include intraepithelial lymphocytes (93), non-adherent splenic cells, adherent phagocytic cells from peripheral blood (52), and possibly heterophils (97,98). A monoclonal antibody marker specific for avian NK cells would be useful in more clearly defining the cells responsible for NK activity; however, no such antibody is currently available.

Specific cell-mediated cytotoxicity

Cells that express certain types of surface molecules (tumor or viral antigens, or allogeneic markers) are killed by mechanisms involving recognition between the target cell and the killing cell. There are two major types of specific cell-mediated killing. One is carried out by cytotoxic T lymphocytes (CTL) which recognize foreign antigens complexed with major histocompatibility (MHC) antigens, usually Class I in type. In the second type, cells bearing receptors for the Fc portion of immunoglobulin molecules (NK cells, macrophages, and granulocytes) recognize specific antibody bound to foreign cells and kill these cells via *antibody-dependent cell-mediated cytotoxicity (ADCC)*.

No information is available on ADCC against avian respiratory pathogens. Avian macrophages have Fc receptors (47,160) and it is surmised that heterophils do, too (131). However, most studies of ADCC in birds have been directed toward tumor cells and non-respiratory pathogens (130,149).

Evidence for CTL-mediated cytotoxicity against avian respiratory pathogens is largely indirect. Positive evidence for a cell-mediated response is considered to be 1) lack of correlation between serum antibody levels and immunity to a particular pathogen and 2) ablation of immunity in thymectomized but not bursectomized birds (122). Although these types of experiments can distinguish between humoral and cell-mediated immunity (CMI), they cannot ascribe cytotoxic activity to specific cell types. For

example, thymectomy may affect either CTL or macrophage responses, since T lymphocytes activate macrophages to become the actual effector cells in some immune responses. Nonetheless, a protective role for CMI has been shown using these types of studies in *Chlamydia psittaci* infections in turkeys (123), *Pasteurella multocida* in chickens (10), and infectious laryngotracheitis virus in chickens (83). Lymphocyte blastogenesis and leukocyte migration inhibition tests indicate a role for CMI in protection of chicken respiratory tract against infectious bronchitis virus (44). Recently developed monoclonal antibodies and *in vitro* assays for effector functions should facilitate studies of CMI in avian respiratory diseases (94).

Antibody-Mediated Avian Respiratory Defense

While cell-mediated immune responses can be of major importance in defending against first encounters with pathogens, antibody-mediated responses more efficiently protect the host in subsequent encounters with the same pathogenic threat. Antibody can act directly by neutralizing toxins and by preventing entry or attachment of pathogens to host cells. It can also act indirectly by coating bacteria or infected cells, thus targeting them for elimination by phagocytosis or ADCC.

Antibody is thought to play an important protective role in several avian respiratory diseases, although much of the experimental evidence is contradictory (reviewed by Powell, 130). In general, positive correlation of

antibody titers with protection, and increased susceptibility to disease after bursectomy are used as experimental evidence supporting a role for antibody-mediated protection. These types of studies have shown antibody responses to be important in limiting infections by infectious bronchitis virus (44,78), Newcastle disease virus, avian influenza virus (48), and *Mycoplasma gallinarum* (186). Experiments using passive transfer of specific immune serum also indicate a role for antibody in immunity to *E. coli* (108) and *Bordetella avium* (8).

Sources of antibody in the avian respiratory tract

Antibody in respiratory secretions is either locally produced by plasma cells in the respiratory mucosa, or it is produced by plasma cells in lymphoid organs and transported via the bloodstream to the respiratory tract (87). In mammals, and presumably birds, all three major immunoglobulin isotypes (IgG, IgM, and IgA) are capable of diffusing in small amounts from serum into airways (87). However, IgG diffuses most readily due to its small size (135) and thus is more abundant in airways than serum-derived IgA or IgM (100). Receptor-mediated transport of IgG across some types of epithelial cells also occurs, effected by binding of the Fc portion of IgG to a specific receptor (103). Whether this occurs in respiratory epithelium or in avian species has not been determined.

The major transport route for polymeric IgA (and, to a lesser degree, IgM) is via binding to the polymeric immunoglobulin receptor (pIgR) on epithelial cells (reviewed in 6). Although best studied in intestinal models, this type of transport also occurs in mammalian bronchial epithelium (84). It is unknown whether a similar process occurs in the avian respiratory tract.

The absolute amount of immunoglobulin on respiratory surfaces varies depending upon species and location within the respiratory tract (87). In mammals, upper respiratory tract secretions contain more IgA than IgG, roughly equal amounts in the bronchi, and a predominance of IgG in the alveoli (136). In birds, the amount of IgG exceeds that of IgA in nasal, tracheal, and tracheobronchial secretions (14,38,51,61). Much of this IgG may be locally produced, since the number of IgG-producing cells is larger than the number of IgA-producing cells in respiratory tract, Harderian gland, and nasal tissues (7,12). This information also suggests that there may be selective transport mechanisms for IgG in birds.

Inflammation can alter the amounts of both antigen-specific and -nonspecific immunoglobulin normally present on mucosal surfaces. For example, in chickens infected with Newcastle disease virus (NDV), there were increased amounts of non-NDV antibodies in tears and saliva. In NDV-vaccinated birds subsequently challenged with NDV, amounts of anti-NDV antibody were significantly increased in tears, saliva, and tracheal washings (2).

Special role of IgA in respiratory defense

The discovery in mammals that IgA is found primarily in secretions and in only low concentrations in serum suggests a special role for IgA in mucosal immunity (reviewed in 103). In birds, the role of IgA is less clear, since the amount of IgG is greater than that of IgA in respiratory secretions, saliva, tears, and semen, while the amount of IgA predominates in bile and intestinal secretions (38,91). Despite the predominance of IgG in tracheal washings, there is a selective enrichment of tracheal IgA; sixteen per cent of the total immunoglobulin from tracheal washings is IgA, compared to five per cent in serum. And IgA:IgG ratios in serum and secretions suggest that most of the IgA in the respiratory tract is locally produced (38). Thus, it would appear that IgA has an important function in the avian respiratory tract even though it is present in lesser amounts than IgG.

Mammalian IgA has several structural features that make it particularly effective in protecting surfaces exposed to the external environment (reviewed in 39). An IgA-associated peptide, the J chain, confers resistance to proteolytic digestion by enzymes often present in mucosal environments. J chain also allows IgA to form polymers which facilitate its binding to the polymeric immunoglobulin receptor (pIgR) for transport across epithelial cells. Part of the pIgR remains associated with the IgA molecule after it is secreted on the epithelial surface and also serves to protect IgA against proteolysis. This remnant of pIgR is referred to as secretory component.

The structure of avian IgA is often assumed to be identical to that of mammalin IgA, however this has not been strictly tested. In chickens a secretory immunoglobulin, called IgA, has been isolated in large amounts from secretions and shown to be different from IgM and IgG (91). This molecule has a J chain (105) and indirect evidence suggests that the molecule binds to secretory component (15). In other respects, however, this secretory immunoglobulin is very different from mammalian IgA. Radioimmunoassay inhibition studies have repeatedly failed to show relatedness of chicken secretory immunoglobulin to IgA from several different mammalian species (4,71). Rather, these studies have shown mammalian IgA to be more closely related to the major chicken serum 7S immunoglobulin, usually referred to as chicken IgG. Until DNA and/or protein sequencing of avian immunoglobulin molecules has been done, assumptions about avian IgA, based on information about mammalian IgA should be made with caution.

Despite the uncertainty about the structure of avian secretory immunoglobulin, the molecule apparently functions in many ways as mammalian IgA does, and thus will be referred to as IgA in this review.

The functions ascribed to mammalian IgA include inactivation of bacterial enzymes and toxins, IgA-mediated ADCC, interference with bacterial attachment to host cells, and virus neutralization; however, the specific contribution of each of these to particular diseases and direct comparisons of IgA-mediated effects to those mediated by IgG or IgM have not been

documented for most mucosal diseases (39). As usual, the data for birds are even sketchier. Two studies on Newcastle disease virus are directly contradictory. One study concluded that locally produced IgA was responsible for 85% of virus-neutralizating activity in tracheal secretions (126). A second study published the same year showed that while neonatal bursectomy resulted in depletion of IgA from secretions, vaccinated and bursectomized birds were as resistant to virus challenge as were vaccinated, sham-bursectomized chickens (51).

Studies of the role of IgA in the immune response to infectious bronchitis virus (IBV) are also difficult to interpret. Neutralizing activity in tracheobronchial secretions after vaccination with a moderate dose of IBV was found to be IgA (61). However, challenge with a higher dose elicited primarily an IgG response in secretions, with a much smaller IgA response. No secretory IgA anamnestic response could be detected and since the IgA response did not reach peak titers until 10-14 days after exposure, it is difficult to ascribe a clear role to IgA in preventing virus infection.

Thus although IgA is generally considered to be the immunoglobulin isotype of primary importance at mucosal surfaces, it remains to be shown that it is important in protecting the avian respiratory tract from pathogenic attack. Since IgA deficiency alone does not result in severe health problems in either humans or birds, it appears that other isotypes can accommodate the protective functions of IgA at mucosal surfaces (41,90). In mammalian species, other

roles for IgA are currently being explored. Secretory IgA has been shown to mediate eosinophil degranulation, (1) and to act as an anti-inflammatory agent by interfering with complement activation (145). IgA may have similar as yet undiscovered functions in birds.

Generation of T-cell Dependent Antibody Responses in the Lung

Regardless of immunoglobulin isotype or site of production, antibody responses to most antigens begin in the same way: with a three-way interaction between an antigen-presenting cell (APC), a T lymphocyte, and a B lymphocyte (66). The role of the APC is to express antigen on its surface in a form which can be recognized by antigen-specific T lymphocytes. Prior to presenting antigen, the APC must internalize it and degrade it into small pieces which associate with a class II major histocompatibility molecule (MHC-II). Processed antigen complexed with MHC-II is then transported and displayed on the APC surface (107). Upon recognition of the antigen-MHC-II complex, T lymphocytes are activated and produce soluble factors (lymphokines) which induce B lymphocytes (previously activated by binding the same antigen to surface immunoglobulin receptors) to proliferate and differentiate into antibody-secreting plasma cells.

The role of various lymphoid tissues in this process is to provide a microenvironment in which T and B cells sensitive to the same antigen are close

enough to interact with each other and the APC (175). The ability of lymphoid tissues in the lung, specifically bronchus-associated lymphoid tissue (BALT), to initiate immune responses to respiratory pathogens must be evaluated in this context.

Again, information about the immunological processes occurring in avian lungs is scarce. However, there is much evidence to support the belief that cellular interactions between APC and T and B lymphocytes are the same in birds and mammals (166).

Avian Lymphoid Tissues

Regional lymph nodes are key elements in mammalian responses to antigens encountered in the respiratory tract (19). However, birds have only a rudimentary system of lymphoid nodules along lymphatic vessels (127). These are small lymphocytic accumulations (usually about 0.5 mm in diameter) which lack a normal sinus structure that would permit large scale filtration of antigens from lymph. While some of these nodules enlarge and develop germinal centers in response to locally injected antigen, they are not of sufficient size or complexity to perform the functions of lymph nodes in mammals (101).

Perhaps in functional compensation for the lack of lymph nodes, birds have an enormous number of lymphoid nodules, complete with germinal centers, in parenchymal tissues such as bone marrow, skin, liver, lung, kidney, pancreas, endocrine glands, peripheral nerves, larynx, and trachea (127). Avian

thymus and bursa, in addition to their classical functions as primary lymphoid organs, are capable of responding to antigens as secondary lymphoid organs (50). Thus birds do not lack lymphoid tissues with the potential to respond to antigens.

Avian Mucosa-associated Lymphoid Tissues

Mucosa-associated lymphoid tissues (MALT) are highly developed in birds and may also perform some of the functions normally ascribed to mammalian lymph nodes. Avian MALT include the Harderian gland, lymphoid nodules associated with the nasolacrimal gland, conjunctiva-associated lymphoid tissue (CALT), bronchus-associated lymphoid tissue (BALT), and lymphoid tissues associated with various elements of the gastrointestinal tract, the gut-associated lymphoid tissues (GALT) (13,55,106). Some of these tissues (e.g., Harderian gland and Meckel's diverticulum of the GALT), contain large numbers of plasma cells; presumably, their primary function is local production of antibody (11,106,117). Other avian MALT, (for example, CALT and GALT), have fewer plasma cells and more lymphoid nodules with germinal centers and thus seem to be involved with the initiation rather than effector phase of antibody production (13,55).

The location of avian MALT is generally similar to that described for mammalian MALT. These tissues develop at sites where exposure to environmental antigens is high. In the respiratory tract BALT is located at

bifurcations in the airways, where increased turbulence in airflow would facilitate deposition of antigenic materials on epithelial surfaces covering lymphoid nodules (22). Mammalian BALT nodules are relatively flat, with a substantial part of the lymphoid tissue lying below the muscularis mucosae (124,151); in chickens, however, most of the lymphoid tissue is above the muscularis, resulting in a nodule that bulges into the bronchial lumen (23). Presumably, this bulging would facilitate interaction of BALT epithelium with antigens, and may partially explain why chicken BALT is more highly developed than its mammalian counterparts (23). In addition to being located at sites favoring exposure to foreign materials, BALT nodules in mammals tend to develop in regions between major arteries and the bronchus lumen (29,59,128). The reason for this is not clear, but seems to be independent of antigen exposure.

Structure/function relationships in BALT

The structure and function of mammalian BALT has been well-studied; however, little is known about the development, cell populations, ultrastructure, and functional capabilities of avian BALT. Although some analogies may be made about structure/function relationships, there are enough differences between mammalian and avian immune systems to warrant independent investigation of avian BALT.

Three main elements comprise BALT as described in mammals: the epithelium, the lymphoid compartment, and the vasculature. Each of these has unique characteristics that contribute to the potential ability of BALT to initiate immune responses.

Epithelium The epithelium that covers BALT nodules is structurally different from the typical bronchial epithelium and has been referred to as a *lymphoepithelium* to reflect the intimate physical association of epithelial cells and lymphocytes within it (56). Normal bronchial epithelium is ciliated, pseudostratified, columnar with a large population of mucus-secreting cells. The combination of mucus and ciliated cells provides an effective barrier from the external environment. BALT epithelium is modified in ways that appear to compromise this barrier. In BALT of rabbits and rats, lymphoepithelial cells (LE) are squamous or cuboidal and non-ciliated (22,56,133,157,172). The apical cytoplasm of LE cells is often attenuated, such that only a thin rim of cytoplasm separates the external environment from subepithelial cells and tissues (22). Mucus-secreting cells are either absent or in low numbers over BALT lymphoid nodules (22,56,110,151,172).

Specializations for uptake of luminal materials occur in BALT epithelial cells. Apical vesicles are present in LE cells of mammalian BALT, especially those in which an active immune response has been stimulated (22,110,133,157,168). Proteins unique to LE of both BALT and GALT have been identified with monoclonal antibodies; these may facilitate uptake of

materials across these tissues (143). Ruthenium red staining of rabbit BALT reveals a distinct glycocalyx on LE cells; this, too, may bind materials prior to uptake (110).

The altered epithelium over BALT nodules has provoked much speculation about its function, with two main hypotheses. One is that the major function of BALT epithelium is in uptake and transport of antigens across the epithelium; the second hypothesis expands this idea to include antigen presentation by LE cells. Numerous experiments have documented the ability of BALT epithelium in rabbits, rats, guinea pigs, calves, and miniature pigs to take up proteins such as horseradish peroxidase and ferritin, as well as particulate materials such as mycobacteria, colloidal carbon, and latex beads (24,56,75,110,157,168). Uptake can be non-specifically enhanced by sensitization, for example by immunization and challenge with mycobacteria (110,168).

The *in vivo* significance of these experiments is unclear, since in guinea pig bronchus, for example, non-LE cells readily take up ferritin and horseradish peroxidase (138). Even in experiments showing preferential uptake of macromolecules by the LE, there is some degree of uptake by non-BALT epithelial cells (168). Since pinocytosis is a constitutive function of most epithelial cells, the difference in uptake between LE cells and other cells of the epithelium may be simply one of degree, with LE cells taking up larger quantities of materials from the lumen. A similar circumstance may exist with the uptake of particulate materials such as bacteria, which are also transported

across non-LE cells of mucosal epithella (179,180). Thus, while transport of both macromolecules and particles occurs across LE cells, this is probably not the sole route of entry for antigenic materials. However, due to the underlying lymphoid cell compartment, transport across the LE may lead to a more immediate immune response, since materials crossing at non-lymphoid sites would have to be carried to sensitized lymphocytes before immune responses could be initiated.

Experiments directly testing antigen presentation by mucosal epithelial cells have generally shown that non-LE cells are more likely to function as antigen-presenting cells. Cultured human bronchial epithelial cells express MHC-II and have been shown to induce T cell proliferation in response to mitogens and CD3 cross-linking, although less effectively than macrophages (104). Other workers have documented the ability of both nasal and bronchial epithelial cells to present antigen to T cells and stimulate mixed lymphocyte reactions in allogeneic systems (86). These studies suggest that unspecialized bronchial epithelial cells are capable of antigen presentation. Studies of GALT have reached similar conclusions; absorptive enterocytes, rather than M cells, constitutively express MHC-II (99) and are able to present antigen and stimulate T cells to proliferate and secrete interleukin-2 (25,85,99,152). In poultry, neither structural or functional studies of BALT epithelium have been done.

Lymphoid compartment The lymphoid compartment of BALT is made up of T and B lymphocytes as well as several types of accessory cells. Five distinct cellular regions can be identified: an area of T and B lymphocytes directly under the epithelium, referred to as the *dome* or *AUE (area under the epithelium)*; B cell *follicles* which may contain germinal centers; a *corona* of small resting lymphocytes adjacent to follicles; a *parafollicular zone* of T cells adjacent to follicles; and an *interfollicular zone* between follicles and at the edge of BALT nodules, comprising specialized vessels and mostly T lymphocytes (110,121,124,151). These regions are analogous to similar regions in lymph nodes and may have similar functions, as well (110,150).

Various immunocytochemical studies have sought to define clear T and B lymphocyte regions within the morphological zones described above (29,43,128,150). However, it is difficult to draw general conclusions from these studies due to the enormous variability resulting from differences in animal species, individual animals, variable immune status, ages of animals, and specificities of monoclonal antibodies used to label cells (128,151). The most accurate generalization that can be drawn is that both B and T lymphocytes are present in BALT nodules, often in localized compartments, and that both T-helper and T-cytotoxic/suppressor cell populations are represented. In poultry BALT, there are distinct T and B lymphocyte regions; however, T cell subsets have not been localized to particular zones within the nodules (7,81).

Accessory cells There are three main types of antigen-presenting accessory cells in lymphoid regions of BALT. *Follicular dendritic cells* (FDC) are present in the dome area and follicles in mammalian BALT (133,150,173) and in germinal centers of chicken BALT (81). These locations are consistent with their function of presenting antigen to B lymphocytes. FDC are distinguished from other types of APC by their lack of MHC-II (141) and by the fact that they handle antigen in the form of unprocessed antigen-antibody complexes (95). They are thought to be important in the maintenance of memory for T dependent humoral immune responses (95).

Interdigitating cells (IDC), which present antigen to T-helper cells, have been localized to parafollicular and interfollicular areas, especially near high endothelial venules in mammalian BALT (29,133,173). In humans and mice, these cells are powerful APC. IDC differ from other APC in that they are loosely adherent to plastic, lack Fc receptors, and are poorly phagocytic. They have a dendritic shape and are strongly MHC-II positive (148).

Macrophages are located throughout the lung parenchyma and BALT of many species including chickens (37,81,169). In rat BALT, macrophages are found between epithelial cells, as well as in dome regions and follicles (37). Enzyme histochemistry and immunocytochemistry have revealed a highly heterogeneous population of macrophages in BALT of rabbits and rats (133,169). Considering the versatile functions of macrophages, their central role in many immune responses, and their capabilities as immune effector cells,

it is not surprising to find that they form a significant and diverse population within BALT nodules of most species.

Germinal Center Formation Germinal centers are a prominent and consistent feature of avian BALT, whereas they are often absent from BALT in mammals (23). The development of germinal centers provides morphological documentation of humoral immune responses (95). What becomes evident from reviewing current literature is that the type of tissue in which germinal centers are found is relevant only with respect to the way in which antigens are delivered (95,175). Spleen receives blood-borne foreign materials; lymph nodes handle antigens transported in lymph draining from regional tissues; and MALT deals with antigens encountered at mucosal surfaces. Beyond these differences, the cells and events in germinal centers are identical. The fact that germinal centers are an integral component of avian BALT supports the idea that this tissue may functionally compensate for the lack of bronchial lymph nodes in birds.

The current model for the events taking place in germinal centers proposes that virgin B lymphocytes first encounter antigen in T cell zones comprised of T helper lymphocytes, B lymphocytes, and interdigitating cells. Antigen-primed B cells then migrate to primary follicles, where they enter the cell cycle to begin a period of exponential cell division. A selection process ensures that only B cells with high affinity for antigen-antibody complexes on follicular dendritic cells are rescued, while all other cells proceed into apoptotic

death. Of the cells surviving the selection process, some receive signals allowing them to leave the cell cycle as small memory B cells, while other cells receive signals inducing them to differentiate into immunoglobulin-producing plasma cells. The entire process is accomplished in about three weeks (95).

The relevance of this model for germinal center development to the development of BALT in avian lungs is by no means established. However, the model is supported by considerable evidence from several mammalian species. The consistency of the evidence suggests that, like the model for cellular interactions between T and B lymphocytes and APC (166), the development of germinal centers is an evolutionarily conserved plan that may be universal among higher vertebrates.

Studies on germinal center development in birds have dealt primarily with responses in chicken spleen. Results of these studies are consistent with the model described above with respect to the cell types involved, the localization of antigen, and morphological changes after antigen challenge (27,80,111,112,161,181,182). The time course for development in chicken mucosal tissues is also consistent with the model; germinal centers appear in nasolacrimal ducts (12), gut-associated lymphoid tissue (81,159), esophageal tonsils (7), and BALT (7,81) between 2 and 3 weeks of age.

Vasculature BALT nodules in species as diverse as horse, rat, and rabbit are consistently located near blood vessels and are highly vascularized (23,37,64,96). Blood vessels are important in the function of BALT from two

standpoints: lymphocyte trafficking through high endothelial venules (HEV) and antigen presentation by endothelial cells.

High endothelial venules are a consistent morphological feature of mammalian BALT (37,128,133). Their existence has not been documented in avian BALT, however they do occur in chicken GALT and CALT (13,55). HEV are located at the periphery of BALT nodules, often at the interface of T- and B-lymphocyte regions. Their structure distinguishes them from other blood vessels in that their endothelial cells are cuboidal rather than flat and they have numerous intraluminal and interendothelial lymphocytes (185). The cuboidal shape of HEV endothelial cells is not an intrinsic characteristic, but is induced by antigenic stimulation and cytokines (129). HEV-associated lymphocytes have been identified as primarily T cells in rat BALT, however some studies have identified large numbers of B lymphocytes in HEV (119, 29).

Receptor/ligand interactions between surface molecules on HEV and lymphocytes are thought to control tissue-specific trafficking and homing phenomena (46). In several mammalian species, lymphocytes originating in bronchial lymph nodes preferentially return to the lung (21,82,153). Similar results have been seen in studies of GALT (82,113,120, 72). It has been proposed that tissue specific *addressins* on endothelial cells and *homing receptors* on lymphocytes mediate the observed migration patterns, and that at least three separate receptor/ligand systems exist - one that mediates tissue-

specific interactions in peripheral lymph nodes, one in MALT, and another for sites of inflammation (113,185).

The idea of tissue-specific interactions between lymphocytes and high endothelial cells of MALT is attractive from the standpoint that cells primed to react to a particular antigen would be attracted back to the tissue in which they would be most likely to re-encounter the same antigen (151). However, homing phenomena are not absolute; substantial numbers of lymphocytes originating in bronchial lymph nodes are also found in intestine and spleen (82,153). Interactions between endothelial cells and lymphocytes are complex, involving many different molecules whose expression may vary depending upon conditions in the local environment (32). Thus, addressins and homing receptors may be only one component in the process of lymphocyte homing to MALT.

Studies of HEV specificity and lymphocyte homing of BALT are in their infancy. In rat tissues, BALT HEV were shown to attract approximately equal proportions of T and B lymphocytes, a pattern similar to that of mesenteric lymph nodes, but different from GALT, in which B cells bind preferentially to HEV (170). Similar types of experiments have not been performed in poultry.

The role of endothelial cells as APC in BALT has not been explored; however, endothelial cells effectively present antigen in several other systems (60). It has been suggested that endothelial cells, expressing antigen and MHC-II on their surfaces, may trap antigen-specific lymphocytes in non-lymphoid

organs, thus initiating local immune responses (60). Interestingly, the first stage in the development of BALT is the accumulation of lymphocytes near vessels in connective tissue of the bronchus (64). This occurs prior to specialization of epithelium, suggesting the possibility that endothelial cells may provide the signal that initiates the tissue modifications ultimately resulting in the formation of a BALT nodule.

Development of BALT

No studies of BALT development in birds have been done. In mammals, however, there are three stages in the development of BALT: *initiation*, characterized by lymphocyte accumulations near the bronchial epithelium; *expansion*, characterized by an increase of number and size of nodules due to lymphocyte division and germinal center formation; and *regression*, characterized by less mitotic activity in lymphoid areas, decreased numbers of nodules and increased amounts of connective tissue. These stages have been documented in rat (64,128), cattle (5), and human BALT (reviewed in 102), and in other MALT in chickens (13,55) and mammals (42,53). Thus they appear to be characteristic of MALT development in general.

Initiation BALT is not found in fetal rats or calves, but appears within the first week of life (5,64). Transplantation of fetal mouse lung to subcutaneous pockets isolated from antigenic exposure does not prevent the development of BALT (24), suggesting that antigen is probably not important

for initiation of BALT lymphoid nodules. While the migration of antigen-containing cells into the BALT precursor tissues was not prevented, the experiments support the idea that BALT develops independently of antigen transported across the bronchial epithelium.

The structure of BALT is reminiscent of that of the thymus with distinct epithelial and lymphocytic compartments. Lymphocytes are attracted to the fetal thymus by factors produced by thymic epithelial cells (139). A recent study showed that bovine bronchial epithelial cells also release factors that are chemoattractive to T and B lymphocytes, suggesting a possible antigen-independent mechanism for the initiation of BALT development (140).

Expansion After sufficient numbers of lymphocytes have entered the bronchial tissues, there is a massive expansion of the lymphocyte population, with subsequent development of germinal centers. Accompanying the expansion of the lymphocyte compartment is an invasion of lymphocytes into the epithelium and alteration of the bronchial epithelium from ciliated to non-ciliated form, although epithelial alteration is not a consistent feature of BALT development (5,64). The mechanism whereby epithelial cells change has not been determined, however it coincides with lymphocyte infiltration (5,23,64,65). Bronchial epithelial cells are dynamic and change from columnar to squamous in form during injury repair, for example (134). It has been suggested that the morphology of intestinal epithelial cells may be altered by lymphokines (34); the same may be true of bronchial epithelial cells.

Keratinocytes, which are very similar ultrastructurally to basal cells of the bronchial epithelium, have been shown to respond to interleukin 1 (IL-1) (26), and factors which induce IL-1 receptors on keratinocytes induce markers of squamous differentiation in skin cells. Thus, lymphocytes may induce the epithelial morphology that enhances access to environmental antigens.

Antigen is important in the expansion stage of BALT development (65). It appears that there is a baseline development of BALT that occurs in all animals as they encounter new environmental antigens. Specific pathogen free rats, for example, develop BALT, but it is not as extensive as that in wild rats which have exposure to vastly more foreign antigens (65). The development of BALT may be accelerated or enhanced by respiratory infections, which are undoubtedly accompanied by increased amounts of foreign antigens (5,65,167). The consistently observed animal-to-animal variation in the degree of BALT development also supports the idea that antigen is important in this stage, as there is genetic variability in the abilities of individual animals to respond to certain antigens (5).

Regression In calves and rats, the number of BALT nodules is decreased in adult animals (5,30). This is similar to findings for GALT in chickens (13) and humans (42). In addition to decreased amounts of MALT in adults, there are also changes in the structure of BALT. There is a loss of follicles from human Peyer's patches with age (42). In conjunctiva-associated lymphoid tissue in chickens, the lymphoepithelium reverts to a more columnar

form with age (55). Whether there are functional losses as well has not been determined.

Respiratory Routes of Immunization

The current model for induction of mucosal immune responses in mammals proposes that antigen from the outside environment is taken up through a lymphoepithelium and presented either by epithelial cells or by underlying macrophages to T lymphocytes, which then activate B lymphocytes. The activated B cells travel to regional lymph nodes and then preferentially return to mucosal sites via addressin/homing receptor interactions. Upon activation by antigen and cytokines produced by T-helper lymphocytes, B lymphocytes differentiate into antibody-secreting plasma cells in the lamina propria of the mucosal tissues in which they originated. Antibody is then secreted and transported across epithelial cells to protect the mucosal surface (20,36). In some systems IgA precursors are thought to be preferentially stimulated by mucosal routes of immunization, however, large numbers of IgG precursors are also generated in mucosal systems (142).

The extent to which this model applies to avian systems is unknown and in many regards untestable, due to the lack of reagents and inbred animals that have facilitated mammalian studies. However, except for the stages occurring in lymph nodes, there are no apparent reasons for the model not to apply to

birds. The events occurring in mammalian lymph nodes may be carried out by other lymphoid tissues, including MALT, in birds.

The model predicts that intratracheal or intrabronchial immunization should be sufficient to induce antigen-specific plasma cells in the respiratory tract. However, experiments of this type have not generally been successful (41). For example, priming of rats via intramuscular injection resulted in more antigen-specific cells in BALT upon subsequent intratracheal challenge than did intratracheal or subcutaneous routes of priming (59). Others, however, found that subcutaneous priming followed by and intratracheal boosting resulted in antigen-specific plasma cells in BALT, while again intratracheal priming followed by intratracheal boosting led to no response (171). In experiments using *Mycoplasma pulmonis* to challenge mice, intratracheal priming followed by intraduodenal challenge resulted in more antigen-specific lymphocyte clones in lung than did intratracheal routes for both priming and challenging (35,142).

In many cases, priming via the oral route is the most effective way to generate protection at respiratory surfaces (16). The reasons for this are not known, but this strategy is widely employed in the poultry industry where vaccines against many respiratory pathogens are delivered in drinking water (116).

Thus, in mammals, much is known about the individual elements of BALT; however, the integration of these elements such that the best route of immunization against respiratory pathogens can be rationally determined has not

yet been accomplished. In birds, the basic elements of the respiratory immune system are still unknown. The simplicity of the avian immune system has served immunology well in the past; perhaps determining how respiratory immune responses are generated in a system lacking regional lymph nodes, but otherwise very similar to that of mammals, can help to unravel the unsolved problems of mucosal immunity.

PART I

A MORPHOLOGIC STUDY OF BRONCHUS-ASSOCIATED LYMPHOID TISSUE IN TURKEYS

A Morphologic Study of Bronchus-Associated Lymphoid Tissue in Turkeys

Jane A. Fagerland and Lawrence H. Arp

From the Department of Veterinary Pathology

Iowa State University, Ames, Iowa 50011

Published in:

The American Journal of Anatomy
1990. 189(1):24-34.

This research was supported by United States Department of Agriculture Grant No. 88-34116-3782; J. Fagerland was a recipient of a Project RENEW Grant from the American Association of University Women.

PART II

STRUCTURE AND DEVELOPMENT OF BRONCHUS-ASSOCIATED LYMPHOID TISSUE IN CHICKENS

Structure and Development of Bronchus-Associated Lymphoid Tissue in Chickens

Jane A. Fagerland and Lawrence H. Arp

From the Department of Veterinary Pathology
Iowa State University, Ames, Iowa 50011

Supported by a grant from the United States Department of Agriculture

SUMMARY

The development of bronchus-associated lymphoid tissue (BALT) in conventional chickens of 1 day, 1, 2, 3, 4, 6, and 8 weeks of age was studied using light and electron microscopy (scanning and transmission). Chicken BALT had many of the characteristics of other mucosa-associated lymphoid tissues, including the presence of an altered epithelium overlying a population of lymphocytes, potential antigen-presenting cells such as macrophages and dendritic cells, and high endothelial venules. In contrast to other MALT, chicken BALT lacked epithelial cells with large numbers of apical tubules and vesicles. There were age-related differences in size, number, and cellular composition of BALT nodules. Lymphoid nodules were progressively larger and more numerous with increasing age through 8 weeks of age. In 1-day and 1-week old chicks, lymphoid infiltrates were covered by a squamous epithelium interrupted by small patches of ciliated cuboidal cells. Epithelial cells of the BALT lymphoepithelium in chickens 2 to 3 weeks of age were primarily non-ciliated squamous or cuboidal, while in chickens 6 weeks of age and older, most lymphoepithelial cells were ciliated columnar. Lymphocyte infiltration of the epithelium was extensive in chickens 1 through 4 weeks of age. The epithelium in these chickens was disrupted, basal cells were displaced, and luminal epithelial cells were attenuated and often appeared degenerate. In chickens 6 weeks of age and older, the basal cell layer of BALT nodules was

uniform, and the luminal surface was lined by a ciliated columnar epithelium interrupted by goblet cells and mucous glands; some nodules were of the less mature type found in younger birds. Epithelial disruption may result in increased permeability of BALT epithelium to luminal antigens and increased accessibility of foreign material to numerous heterophils and macrophages in BALT.

Respiratory diseases result in substantial economic loss to the poultry industry (1). However, the immune response to avian respiratory pathogens is poorly understood and the tissue sites at which avian immune responses to mucosal antigens occur have not been precisely determined.

Studies in turkeys have shown that lymphoid nodules comprising the bronchus-associated lymphoid tissue (BALT) are more numerous and widely distributed in Bordetella avium-infected turkeys, suggesting a role for BALT in respiratory immunity (18). Also in turkeys, age-related changes in the structure of BALT lymphoepithelium (LE) may have important functional consequences, particularly with regard to accessibility of antigen to underlying lymphoid cells (6).

Chickens possess BALT (3), but systematic studies of its structure and development have not been reported. The aim of this study was to characterize the morphological development of BALT in conventional chickens from 1 day to 8 weeks of age and to compare it to BALT previously described in turkeys (6).

MATERIALS AND METHODS

Forty-five broiler chicks were obtained from Hoover's Hatchery (Rudd, Ia.) and housed in brooder cages with free access to food and water. At 2 weeks of age, the chicks were moved to pens on a concrete floor. At 1 day, 1, 2, 3, 4, 6, and 8 weeks post-hatching, chickens were euthanatized with an intraperitoneal or intravenous injection of sodium pentobarbital.

Lungs were fixed immediately by intratracheal perfusion as previously described (6). Lungs from three chickens of 1 day and 1 week of age and from five chickens of each age from 2 - 8 weeks were fixed in 10% neutral buffered formalin for light microscopy (LM). Lungs from two additional chickens of each age were fixed in 3% glutaraldehyde in 0.075 M Sorensen's phosphate buffer, pH 7.3, for scanning and transmission electron microscopy.

Tissues for LM were routinely processed, dehydrated in ethanol, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) for general morphological evaluation, Gomori's trichrome for connective tissue, and periodic acid Schiff's/Alcian blue (PAS/AB) for mucus-producing cells. Serial sections were made of the entire primary bronchus in 1-day old chicks. Every fifth section was stained with H&E and examined for presence of BALT.

Tissues for transmission electron microscopy were post-fixed in 1% osmium tetroxide, dehydrated in ethanol and propylene oxide, and infiltrated with epoxy resin (EMbed 812, Electron Microscopy Sciences, Ft. Warrington,

PA). Thin-sections were cut and stained for 10 minutes each with 2% methanolic uranyl acetate and Reynolds' lead citrate.

For scanning electron microscopy (SEM), tissues were rinsed vigorously with 0.075 M Sorensen's phosphate buffer, pH 7.2, to remove mucus, fixed overnight in glutaraldehyde/tannic acid, then treated sequentially with several changes of osmium tetroxide and tannic acid to improve conductivity and secondary electron emission, using the method of Sweney and Shapiro (16). Tissues were then dehydrated in a graded ethanol series, critical point dried from absolute ethanol, and sputter-coated with gold-palladium.

RESULTS

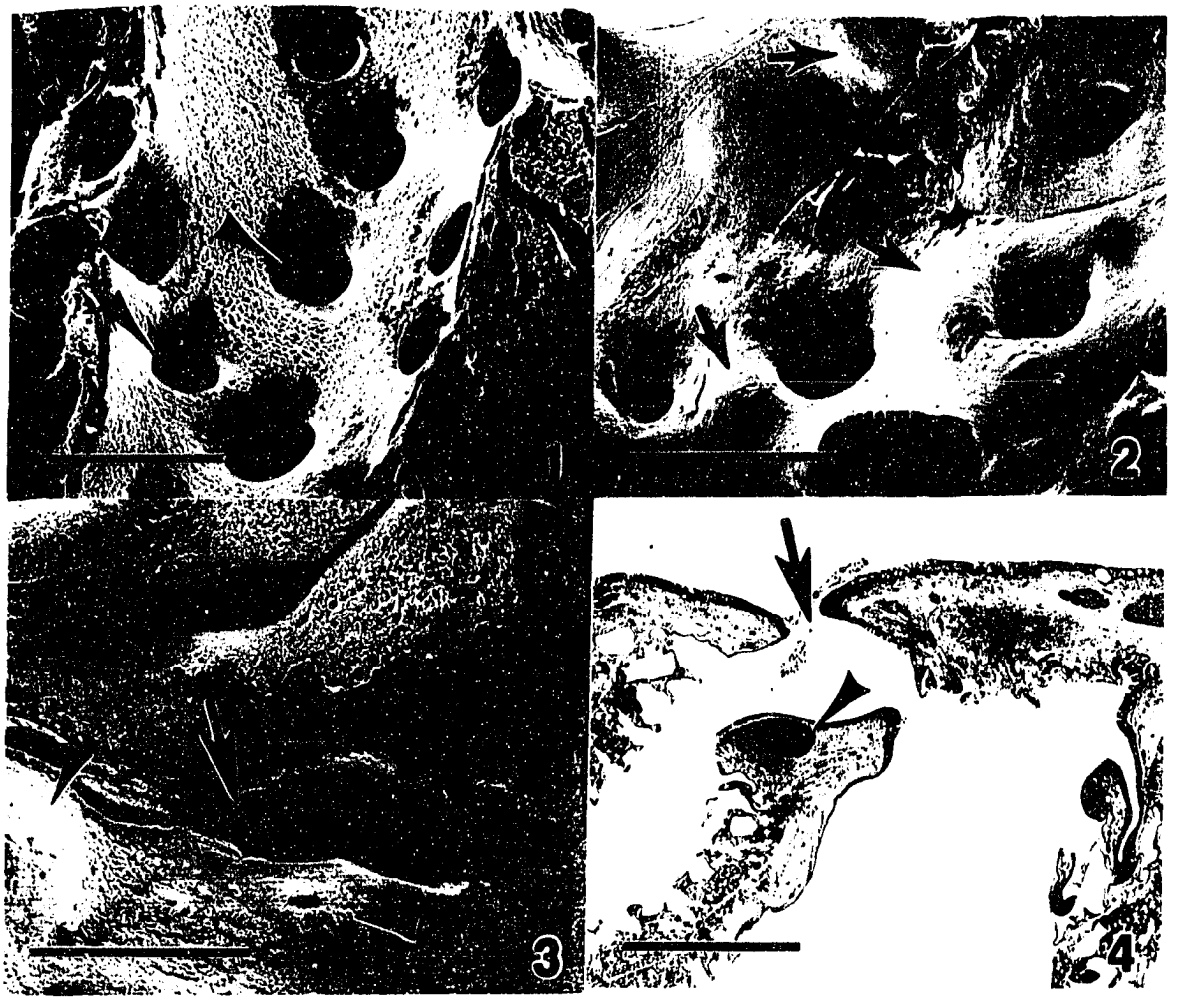
In chickens of the same age, the size, number, and structure of BALT nodules varied slightly from bird to bird. Despite this variability, a progression of age-related changes could be defined with respect to epithelial cell type, basal layer integrity, and organization of underlying lymphoid tissue.

General structure of BALT Number and size of nodules increased with increasing age from 1 day through 8 weeks post-hatching. In 1-day old chicks, single small peribronchial lymphocyte infiltrates were found in two of the three birds examined with LM; there were no nodules apparent in SEM preparations (Figure 1). However, in 6- and 8-week old chickens nearly every bronchial opening was surrounded by a ring of lymphoid tissue (Figure 2).

In 4-week old chickens, there were small folds in the primary bronchial epithelium at secondary bronchial openings. Pockets of lymphoid tissue were associated with these folds in 6-and 8-week old chickens (Figures 3 and 4).

Epithelial component of BALT The primary cell type of BALT lymphoepithelium (LE) changed from squamous and ciliated cuboidal in 1-day old chicks to ciliated columnar in older birds (Figures 5-8). In 1-day old chicks, patches of non-ciliated cells covered lymphoid infiltrates, but the basal cell layer was continuous and most lymphocytes were subepithelial (Figure 5). In nodules of 1-, 2-, and 3-week old chickens, infiltration of lymphocytes into the LE was marked; the epithelium often appeared to be greatly thickened due to the

Figs. 1-4. 1. Primary bronchus with openings to secondary bronchi (arrowheads) in 1-day old chick. Bar = 500 μm. 2. Primary bronchus of 6-week old chicken with numerous lymphoid nodules (arrows) around openings to secondary bronchi. Bar = 1 mm. 3. Pocket-like invagination of epithelium (arrow) around opening to secondary bronchus in 6-week old chicken. Arrowheads indicate lymphoid nodules. Bar = 500 μm. 4. Section through area similar to that in previous figure. Large arrow is in opening of secondary bronchus; arrowhead indicates lymphoid tissue with germinal center in pocket-like region. Bar = 1 mm.


2
3
4

Figs. 5-8. 5. Primary bronchus of 1-day old chick with subepithelial lymphocytic infiltrate. With exception of small patch of ciliated cells (arrowheads), epithelium is non-ciliated. Bar = 200 μm. 6. BALT nodule in primary bronchus of 2-week old chicken. Epithelium is irregular and composed of non-ciliated squamous cells. There is extensive invasion by lymphocytes. Arrowhead indicates early germinal center. Bar = 200 μm. 7. BALT nodule in primary bronchus of 4-week old chicken. Epithelium is ciliated low columnar with a small non-ciliated patch (arrowheads). Basal cells (arrows) form an intact layer under ciliated cells. Bar = 200 μm. 8. BALT nodule in primary bronchus of 6-week old chicken. Epithelium is tall ciliated columnar. Bar = 200 μm.

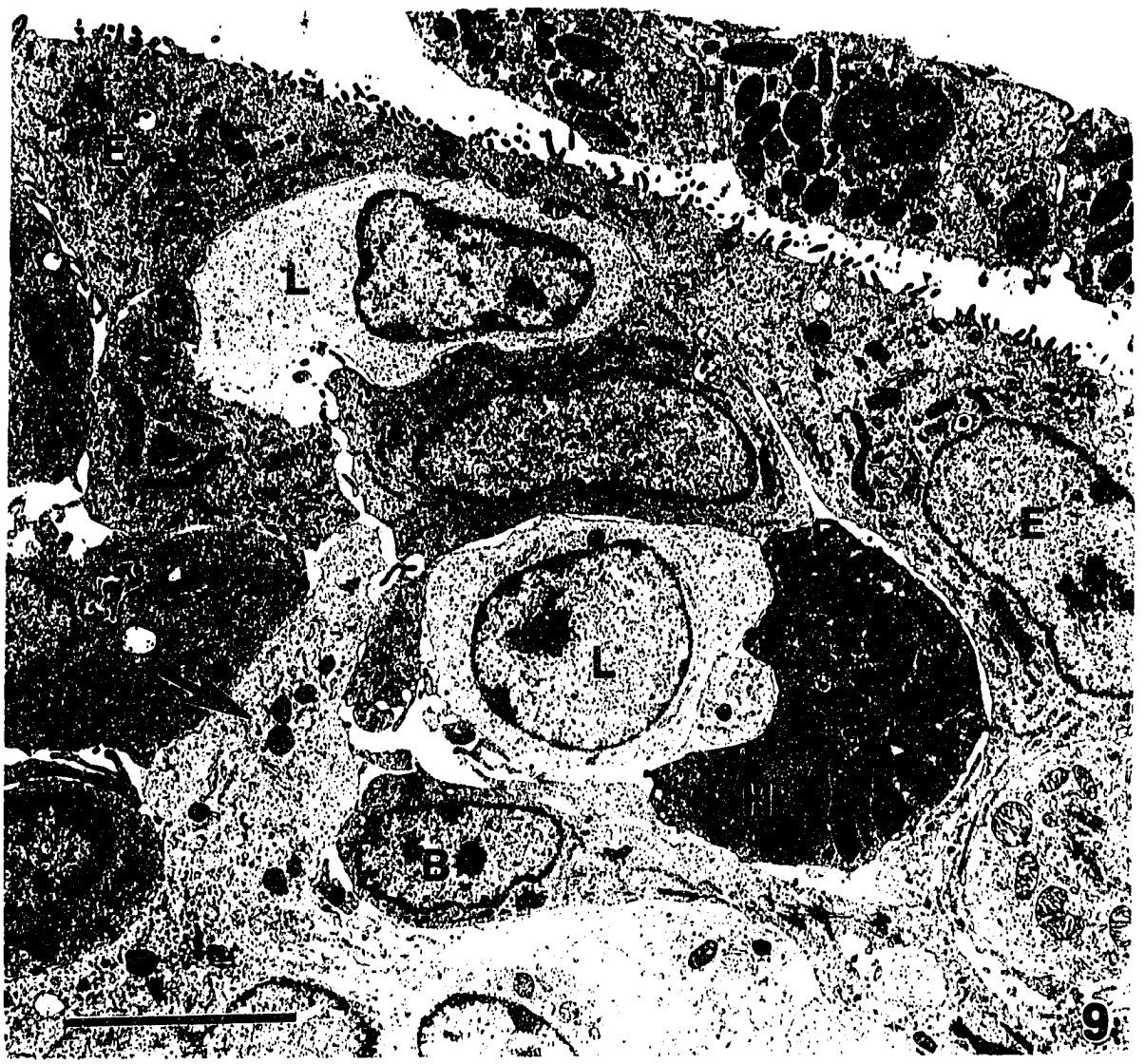

5
6
7
8

number of intraepithelial lymphocytes. Cell processes of lymphocytes and macrophages extended between basal cells of the LE, displacing basal cells from their normal orientation. The apical cytoplasm of epithelial cells was attenuated (Figure 9). In 2-and 3-week old chickens, LE cells were primarily non-ciliated; many were highly eosinophilic, shrunken, and appeared degenerate (Figure 6). By SEM, the LE surface was irregular, with depressions and small pits.

At 4 weeks, some of the nodules resembled those of younger chickens, while others lacked large intraepithelial lymphocyte populations and were covered with an epithelium that was primarily ciliated with small non-ciliated patches (Figure 7). In 6- and 8-week old chickens, the LE was similar to that in 4-week old birds except that the ciliated cells were usually of a high columnar type and there were fewer non-ciliated patches (Figure 8). Basal epithelial cells of the LE in 4-, 6-, and 8-week old chickens formed a uniform layer along the basal lamina, interrupted only where there were small regions of lymphocyte infiltration into the epithelium.

With TEM, four types of non-ciliated epithelial cells could be distinguished in the LE: (1) morphologically unspecialized cells with irregularly arranged microvilli (Figure 9); (2) immature ciliated cells with basal bodies; (3) secretory cells with large apical granules; and (4) neuroepithelial cells having small (100 nm diameter) electron-dense granules. Neuroepithelial cells were seen in chickens from 1 day through 3 weeks of age, usually at the periphery of BALT

Fig. 9. Lymphoepithelium of 1-week old chicken. Cytoplasmic process (arrow) of cell from lamina propria between basal epithelial cells. E = epithelial cells with irregular microvilli. L = lymphocytes. H = heterophils. B = basal epithelial cell with desmosomes and tonofilaments. Bar = 5 μm.


E
L
L
E
B
9

nodules. None of the 4 types of non-ciliated cells had an abundance of apical pits or tubules. The LE was devoid of mature mucous cells until 6 weeks of age.

Lymphoid component of BALT Subepithelial aggregates of small lymphocytes were first seen in 1 day-old chicks as perivascular infiltrates. Small lymphocytes were in close contact with long cytoplasmic processes of cells that appeared to be macrophages.

In 2- to 4-week old chickens, lymphocytes were organized into germinal centers which had distinct zones: a dark zone comprising tightly packed lymphoblasts, many in mitosis; and a lighter zone of less compressed lymphoblasts and tingible body macrophages. A diffuse mantle-like zone of small lymphocytes enveloped the germinal centers. There were multiple germinal centers in most nodules of 6- and 8-week old birds.

An occasional plasma cell was seen under the epithelium in 1-week old chickens. In chickens 3-weeks of age and older, large numbers of plasma cells were both in and directly under the LE and at BALT nodule edges.

Other cell types and tissue components Connective tissue associated with lymphoid nodules in BALT was poorly developed in younger chickens. In 1-day and 1-week old chicks, collagen was associated only with the smooth muscle layer under the bronchial lamina propria. At 2 and 3 weeks of age, there was a fine collagen stroma at the nodule periphery, surrounding blood vessels, and under the epithelium.

Connective tissue was more prominent in older chickens. A distinct sheath of collagen and fibroblasts partially invested germinal centers in 4-week old chickens. This connective tissue sheath completely encircled germinal centers in 6- and 8-week old chickens, isolating lymphocyte and epithelial cell compartments.

Typical high endothelial venules were at the periphery of nodules in chickens 2 weeks through 8 weeks of age.

Chicken BALT contained large numbers of heterophils, dendritic cells, and macrophages. Heterophils were in the LE, on the bronchial surface, and in vessels in BALT nodules in all chickens. In subepithelial lymphoid aggregates and at the periphery of nodules, cells with long dendritic processes were intimately associated with lymphocytes in BALT from all ages examined.

Location of lymphoid nodules Lymphoid nodules were restricted to openings of the three caudalmost sets of secondary bronchi (the mediodorsal, lateroventral, and laterodorsal) in all tissues examined. There were no lymphoid nodules at the openings to the cranialmost set of secondary bronchi (the medioventral) or along longitudinal ridges and furrows in the cranial portion of the primary bronchus.

In addition to lymphocyte infiltrates associated with dome-shaped nodules at the secondary bronchial openings, large aggregates of lymphocytes occurred elsewhere. In 2-week old chickens, there were perivascular lymphocyte

accumulations in the submuscularis; germinal centers were seen in similar infiltrates in 6-week old chickens.

Many of the secondary bronchi contained large lymphoid nodules with germinal centers, usually near points of bifurcation from the primary bronchus. Smaller subepithelial lymphoid infiltrates were seen in a few parabronchi and atria in tissues from chickens of all ages.

Other observations Chickens were clinically normal during the course of these studies and there were no lesions of respiratory disease in tissue sections. Many of the chickens did, however, have varying degrees of mild pulmonary inflammation. Histopathological changes included inflammatory cell infiltrates (primarily heterophils or eosinophils), mild submucosal edema, and occasional peribronchial granulomas.

DISCUSSION

The location, structure, and development of BALT in chickens is similar to that of turkeys (6). In both, lymphoid nodules are located only at the caudalmost openings to the secondary bronchi. This pattern of localization supports the idea that antigen is as important in the development of BALT as it is in the development of other mucosa-associated lymphoid tissues (5). In birds, inspired air containing foreign materials bypasses the medioventral secondary bronchi and enters the three caudal sets of secondary bronchi first (19). Turbulence created at these openings facilitates deposition of heavier foreign materials on the epithelium (7). It is not until after passing through the filtering mechanisms of the lung and air sacs that air passes through the medioventral openings, which do not develop BALT. Invagination of the epithelium around secondary bronchial openings (Figures 3-4) presumably would also enhance antigen-epithelium interactions.

Cells corresponding to M-cells of other mucosa-associated lymphoid tissues are lacking in the LE of normal chicken and turkey BALT. Altered BALT LE cells in turkeys and chickens have attenuated cytoplasm which may compromise the barrier that normally exists to protect tissues from the environment, but cell organelles such as apical vesicles and tubules for antigen uptake are not present. Rather than being inherently modified for a specific function, altered cells overlying avian BALT nodules may simply represent the effects of

cytokines on normal bronchial epithelial cells, or responses to mechanical stresses created by a burgeoning population of infiltrating lymphocytes.

The changes in epithelial cell type and the ages at which they occur are similar in chickens and turkeys, perhaps reflecting exposure to new antigens. In both, an early period of intense lymphocyte infiltration and epithelial modification is followed by restoration of a more typical ciliated bronchial epithelium and separation of epithelial and lymphocyte cell compartments by connective tissue. Changes such as cell attenuation, lack of cilia, and surface discontinuities would be expected to increase epithelial permeability to antigens present in the bronchial lumen, particularly in 1- to 3-week old birds.

The large numbers of heterophils in BALT of both chickens and turkeys suggests they have an important role in avian respiratory defense and immunity, especially since birds lack a large resident pulmonary macrophage population (17). Studies in avian and mammalian mucosal systems raise the possibility that macrophages and granulocytes in BALT could function by transporting materials captured in the bronchial lumen to sites capable of initiating specific immune responses (9,15). Thus, two mechanisms of handling foreign materials - passage between epithelial cells and transport in phagocytic cells - may actually occur, since phagocyte transmigration alone has been shown to increase epithelial permeability (12). Our laboratory is currently studying possible routes of antigen uptake using tracer substances.

Studies correlating the fate of antigens administered in the respiratory tract with the development of specific immunity have been done primarily in mammals. These studies have shown that translocation of antigen from lungs to draining lymph nodes is a necessary step in the generation of specific immune responses (2). Avian lymph node analogues, however, are small aggregates of lymphocytes in the walls of lymphatic vessels (4). Although certain of these lymphoid aggregates enlarge in response to locally administered antigen (11), most are small nodules lacking sinusoids to provide the filtering function ascribed to mammalian lymph nodes (8). Perhaps to compensate for the lack of organized lymph nodes, birds generate large numbers of lymphoid nodules, complete with germinal centers, in tissues and organs throughout the body (10). Since the structure of avian BALT germinal centers is similar to that described for mammalian lymph node germinal centers (13), it is conceivable that germinal centers in peripheral lymphoid nodules, including those in BALT, could carry out some of the same functions as mammalian lymph nodes; i.e., initiation of immune responses, generation of memory cells, and affinity maturation of antibody responses. Studies of post-bursal somatic mutations in avian immunoglobulin genes have hinted that peripheral lymphoid tissues may be involved in fine-tuning the humoral immune response (14,20).

REFERENCES

1. American Association of Avian Pathologists Poultry Health Research Priorities. American Association of Avian Pathologists, Kennett Square, PA. 1989.
2. Bice, D. E., M. A. Degen, D. L. Harris, and B. A. Muggenburg. Recruitment of antibody-forming cells in the lung after local immunization is nonspecific. Am. Rev. Resp. Dis. 126:635-639. 1982.
3. Bienenstock, J., N. Johnston, and D. Y. E. Perey. Bronchial lymphoid tissue. I. Morphologic characteristics. Lab. Invest. 28:686-692. 1973.
4. Biggs, P. M. The association of lymphoid tissue with the lymph vessels in the domestic chicken (Gallus domesticus). Acta Anat. 29:36-47. 1957.
5. Cornes, J. S. Number, size, and distribution of Peyer's patches in the human small intestine. Gut 6:225-233. 1965.
6. Fagerland, J. A., and L. H. Arp. A morphologic study of bronchus-associated lymphoid tissue in turkeys. Am. J. Anat. 189:24-34. 1990.
7. Fedde, M. R. Anatomic and physiologic features of the avian respiratory system. Proceedings of the 40th North Central Avian Disease Conference and Poultry Respiratory Disease Symposium, Minnesota Extension Service, University of Minnesota, St. Paul MN, pp. 4-8. 1989.

8. Firth, G. A. The normal lymphatic system of the domestic fowl. Vet. Bull. 47:167-179. 1977.

9. Harmsen, A. G., M. J. Mason, B. A. Muggenburg, N. A. Gillett, M. A. Jarpe, and D. E. Bice. Migration of neutrophils from lung to tracheobronchial lymph node. J. Leuk. Biol. 41:95-103. 1987.

10. King, A. S., and J. McLelland. Birds: Their Structure and Function. Bailliere Tindall, London. pp. 229-236. 1984.

11. McCorkle, F. M., R. S. Stinson, I. Olah, and B. Glick. The chickens's femoral-lymph nodules: T and B cells and the immune response. J. Immunol. 123:667-669. 1979.

12. Milks, L. C., G. P. Conyers, and E. B. Cramer. The effect of neutrophil migration on epithelial permeability. J. Cell Biol. 103:2729-2738. 1986.

13. Nieuwenhuis, P., and D. Opstelten. Functional anatomy of germinal centers. Am. J. Anat. 170:421-435. 1984.

14. Parvari, R., E. Ziv, F. Lantner, D. Heller, and I. Schechter. Somatic diversification of chicken immunoglobulin light chains by point mutations. Proc. Natl. Acad. Sci. USA. 87:3072-3076. 1990.

15. Popiel, R., and P. C. B. Turnbull. Passage of Salmonella enteritidis and Salmonella thompson through chick ileocecal mucosa. Infect. Immun. 47:786-792. 1985.

16. Postek, M. T., K. S. Howard, A. H. Johnson, and K. L. McMichael. Scanning Electron Microscopy. Ladd Research Industries, Burlington, Vermont. p. 293. 1980.

17. Toth, T. E., and P. B. Siegel. Cellular defense of the avian respiratory tract: paucity of free-residing macrophages in the normal chicken. Avian Dis. 30:67-75. 1985.

18. Van Alstine, W. G., and L. H. Arp. Histologic evaluation of lung and bronchus-associated lymphoid tissue in young turkeys infected with Bordetella avium. Am. J. Vet. Res. 49:835-839. 1988.

19. Wang, N., R. B. Banzett, J. P. Butler, and J. J. Fredberg. Bird lung models show that convective inertia effects inspiratory aerodynamic valving. Respir. Physiol. 73:111-124. 1988.

20. Weill, J. C., C. A. Reynaud, O. Lassila, and J. R. L. Pink. Rearrangement of chicken immunoglobulin genes is not an ongoing process in the embryonic bursa of Fabricius. Proc. Natl. Acad. Sci. USA. 83:3336-3340. 1986.

ACKNOWLEDGMENTS

The authors wish to thank Theresa Holderread, Traci Koeppel, Diane Nelson, and Becky Smith for technical assistance. This research was supported by United States Department of Agriculture Special Research Grant No. 88-34116-3782.

PART III

DISTRIBUTION AND QUANTITATION OF PLASMA CELLS, T LYMPHOCYTE SUBSETS, AND B LYMPHOCYTES IN BRONCHUS-ASSOCIATED LYMPHOID TISSUE OF CHICKENS: AGE-RELATED DIFFERENCES

Distribution and Quantitation of Plasma Cells, T Lymphocyte Subsets, and B Lymphocytes in Bronchus-Associated Lymphoid Tissue of Chickens: Age-Related Differences

Jane A. Fagerland and Lawrence H. Arp

From the Department of Veterinary Pathology
Iowa State University, Ames, Iowa 50011

Supported by a grant from
the United States Department of Agriculture

ABSTRACT

In this study development of the lymphoid cell compartment in bronchus-associated lymphoid tissue (BALT) of specific pathogen free chickens was examined. Specifically, IgA-, IgG-, and IgM-producing plasma cells, B lymphocytes, and T cell subsets (CD4 and CD8) were labeled with antibodies, using immunocytochemical methods. Immunoglobulin-producing cells (IgPC) were quantitated and comparisons made among chickens of different ages, among immunoglobulin isotypes, and between lymphoid and non-lymphoid areas. At hatching, chickens were devoid of IgPC of any isotype. At 1 week of age, there were IgG+ cells; and by 2 weeks of age all three isotypes were present in BALT. At all ages, IgPC were more numerous in non-BALT regions of the primary bronchus than in BALT regions. At 2 weeks of age, there were approximately equal numbers of IgA-, IgG-, and IgM-PC; thereafter, IgG- and IgM-producing cells (IgM-PC) outnumbered IgA-PC. There were very few T and B (CB3+) lymphocytes in 1 day-old chicks; but by 1 week of age there were small accumulations of T cells under BALT epithelium, and by 2 weeks there were CB3+ cells in BALT nodules. In general, T helper (CD4+) cells were near B cell regions, while T cytotoxic/suppressor (CD8+) cells were more evenly distributed throughout lymphoid nodules and in the epithelium. CB3+ B lymphocytes predominated in germinal centers and overlapped CD4+ populations in parafollicular regions. The findings of this study support a role

for BALT in initiation and/or regulation of respiratory immune responses in chickens.

INTRODUCTION

It has been proposed that antibody-mediated defense of surfaces such as those lining the respiratory tract is generated by mucosal exposure to pathogens, resulting in local priming of immunocompetent cells. Primed cells then proliferate to form a population capable of mounting an effective mucosal immune response on subsequent exposure to the same pathogen (1). However, practical application of this principle (e.g., in mucosal vaccines) has proved to be more complicated than simply exposing mucosal sites to immunogens (2). Because respiratory disease in poultry is of major economic importance, and vaccination via an aerosol route would be a practical method to protect large flocks of chickens and turkeys, we have been investigating the role of bronchus-associated lymphoid tissue (BALT) in the avian respiratory immune response.

Indirect evidence supports the idea that BALT may be important in respiratory defense. In chickens and turkeys, BALT nodules are located at branchpoints of the primary and secondary bronchi (3,4), where air flow patterns favor deposition of foreign materials on BALT epithelium (5,6). The number of BALT nodules increases in some avian respiratory diseases (7), also suggesting a role for BALT in immune responses to respiratory pathogens.

This study describes the development of BALT in specific pathogen free (SPF) chickens and uses immunocytochemistry to document the distribution

pattern of B lymphocytes and T lymphocyte subsets in chickens of different ages, as well as the numbers of immunoglobulin-producing cells of three isotypes (IgG, IgM, and IgA) in BALT and non-BALT regions of the primary bronchus.

MATERIALS AND METHODS

Animals Specific pathogen free chickens (RF-II) were obtained from Hy-Vac Laboratory Eggs Company (Gowrie IA 50543 USA). Eggs from a flock continuously monitored and certified to be free of 22 known pathogens, including 7 common respiratory agents, were hatched in isolation in a room with filtered air and positive pressure. From this hatching, birds were shipped to us at 1-day, 1-, 2-, 3-, 4-, 6-, and 8-weeks of age. On the day of shipment, chickens were euthanatized by sodium pentobarbital overdose and both lungs were collected from six birds at each age. Three sets of lungs were formalin-fixed for staining of plasma cells, and three sets were frozen for staining of T and B lymphocytes.

Tissue Collection and Fixation For evaluation of plasma cells, lungs were fixed *in situ* by intratracheal perfusion with 10% neutral buffered formalin. When abdominal airsacs were full and fixative could be seen in the trachea, lungs were removed from the birds, opened to expose the primary bronchus, and placed in fresh formalin for 4 hours. Because in pilot studies we found that retention of antigenicity depended upon the duration of formalin-fixation, we adhered strictly to the 4-hour fixation time and held tissues in 70% ethanol until they could be routinely processed for paraffin-embedding. Sections were cut at 3 μm, collected on poly-L-lysine coated slides, incubated in a 37°C oven overnight, and stored at room temperature until needed. For morphological

evaluation of BALT development, some sections were stained with hematoxylin and eosin.

For T and B lymphocyte labeling, fresh lungs were removed from euthanatized chickens, and dissected to expose the primary bronchus. Tissues were immediately embedded in OCT (Miles Inc., Elkhart IN 46515 USA) and frozen in liquid nitrogen, then stored at -70°C. Frozen sections were cut at 8-10 μm, fixed in acetone for 5 minutes at room temperature, incubated for 20 minutes at 4°C in a cryoprotective storage medium (42.80g sucrose; 0.33g $MgCl_2$; 250ml phosphate buffered saline, pH 7.2; 250ml glycerol), then stored in slide boxes at -70°C until needed.

A variety of fixatives were used in unsuccessful attempts to preserve T cell markers. These fixatives included acetone/methyl benzoate/xylene (8); Omni-Fix II, an ethanol/ethylene glycol/acetic acid/$ZnCl_2$ fixative (An-Con Genetic, Inc.; Melville NY 11747 USA); periodate-lysine-paraformaldehyde (9), dimethylsuberimidate (10), ethylacetimide (11), and ethylcarbodiimide (12). There was no positive staining in paraffin-embedded tissues fixed with any of these solutions.

Antibodies Goat-derived polyclonal antibodies specific for chicken immunoglobulin heavy chains (Bethyl Laboratories, Montgomery TX 77356, USA) were used to identify immunoglobulin-producing cells; these antibodies were used at dilutions of 1:2000 for IgM and IgA and 1:4000 for IgG. Monoclonal antibodies against CB3, a non-immunoglobulin B lymphocyte marker

(13), and against the T-lymphocyte associated molecules CD3 (found on all T lymphocytes), CD4 (a T helper lymphocyte marker), and CD8 (a cytotoxic T lymphocyte marker) (14,15) were kindly supplied by Dr. Chen-Lo Chen (University of Alabama, Birmingham AL 35294 USA). Antibodies to T cell markers were used at dilutions of 1:10; the B cell marker was used at 1:20.

Biotinylated secondary antibodies were used at a dilution of 1:200 in all procedures. Rabbit anti-goat serum (Vector Laboratories, Burlingame CA 94010 USA) was used for plasma cell staining; horse anti-mouse serum from the same commercial source was used for T and B lymphocyte staining.

Staining Protocols For plasma cell labeling, deparaffinized and rehydrated tissue sections were treated for 5 minutes in 3% H_2O_2 to block endogenous peroxidase in erythrocytes and heterophils, followed by 0.1% trypsin (in 0.05M Tris, pH 7.6, containing 0.1% $CaCl_2 \cdot 2H_2O$) for 2 minutes to expose antigenic sites. Non-specific binding of antibodies was blocked by incubating tissue sections for 10 minutes in 5% normal rabbit serum diluted in Tris/PBS (5mM Tris pH 7.6, 9mM phosphate-buffered saline, pH 7.2). Incubations in primary and secondary antibodies were for 15 minutes each. Streptavidin-horseradish peroxidase (Dako Corp., Carpinteria CA 93013 USA), diluted 1:80 in Tris/PBS, was applied to slides for 10 minutes at room temperature followed by 7 minutes in an aminoethylcarbazole (AEC) and hydrogen peroxide substrate (16). All incubations were at room temperature in

humidified chambers and all washes between incubations were in 0.05 M Tris, pH 7.6.

For T lymphocyte labeling, frozen sections were rehydrated in 0.01M phosphate-buffered saline (PBS), pH 7.2 for 5 minutes, then blocked for endogenous peroxidase using 0.3% hydrogen peroxide in PBS for 10 minutes. Non-specific binding of antibodies was blocked with a 1:20 dilution of normal horse serum containing 5% bovine serum albumin. Incubations in primary and secondary antibodies and streptavidin-horseradish peroxidase were for 30 minutes each at room temperature. Incubation in AEC substrate was for 10 minutes.

The staining protocol for B lymphocytes was the same as for T lymphocytes except that all washes were in Tris/PBS (1:10) containing 0.25 M sodium chloride to reduce excess background staining that occurred with the CB3 monoclonal antibody. Both frozen and paraffin-embedded sections were counterstained with Shandon Instant Hematoxylin (Shandon Inc., Pittsburgh PA 15275 USA).

Positive and negative control slides for each primary antibody were included in every batch of sections stained. Negative controls consisted of incubating comparable lung sections in Tris/PBS instead of primary antibody, with all other incubations and washes unchanged. Positive controls were frozen sections of spleen for the CB3 B lymphocyte marker and frozen sections

of thymus for the three T cell markers. Known positive lung sections were used as positive controls for immunoglobulin markers.

Quantitation The number of IgA-, IgG-, and IgM-producing cells per unit area of tissue was calculated using tissue sections from three chickens at each age. Mucosal areas between the luminal surface of the epithelium and the edge of the muscularis mucosae were computed using a Zeiss SEM-IPS image analysis system (Zeiss-Kontron:IBAS version 2.00; Carl Zeiss, Inc. Thornwood NY 10594 USA). Sony UP5000 Videoprints (Sony Corp. of America, San Diego CA 92127 USA) provided records of the regions for which areas were calculated and served as guides for cell counting which was done using a light microscope.

Numbers of cells per unit area were compared between ages, isotypes, and BALT versus non-BALT regions. In statistical analyses, the experimental unit was an individual bird, euthanatized at a given age. A completely randomized design was used; the regions within a bird (BALT and non-BALT) and the antibody isotype within a region were considered subplots of the bird.

RESULTS

BALT Development BALT in SPF chickens consisted of epithelium-covered nodules of lymphocytes (Fig. 1). These nodules were located in the primary bronchus at the openings of the secondary bronchi. The size and number of lymphoid nodules increased with age, as did the amount of tissue separation between epithelial and lymphocytic cell compartments. Lymphocytic infiltrates in the primary bronchus were seen in only one of the six 1-day old birds examined; however, by 1 week of age, there were lymphocytic infiltrates in all six chickens examined.

Lymphocytes and epithelial cells formed a true lymphoepithelium, with intimate contact between the two cell types, in many nodules from 1-week through 4-week old chickens. Beginning at 4 weeks of age increasing amounts of connective tissue separated epithelial and lymphocytic cellular compartments. Epithelial cells covering nodules in 1- to 3-week old birds were often nonciliated and cuboidal or squamous. In older birds, especially those with substantial amounts of connective tissue between the epithelial and lymphoid tissues, the epithelial cells were primarily ciliated columnar. Germinal centers were first seen at 3 weeks of age.

Other features of BALT lymphoid nodules were the presence of high endothelial venules, which were especially prominent in 2-week old chickens. Heterophils were consistently seen in lymphoid nodules both within the

Figure 1. BALT nodule from 3-week old chicken. Extensive lymphocyte infiltration of epithelium (between arrows). More typical bronchus epithlium is on sides of nodule. Note absence of goblet cells in epithelium. Early germinal center is indicated by arrowhead. Formalin-fixed tissue, hematoxylin and eosin-stained. Bar = 100 μm.

epithelium and in the lamina propria. Goblet cells were absent or reduced in numbers in the epithelium over lymphoid nodules.

Immunoglobulin-Producing Cells There were no immunoglobulin-producing cells of any isotype in 1-day old chicks. At 1 week of age, there were IgG+ cells; and by 2 weeks IgA+, IgG+, and IgM+ cells were present in all birds.

There were mature plasma cells of all isotypes directly under and sometimes within the epithelium of both BALT and non-BALT areas (Fig. 2a, 2b). In BALT nodules, IgPC were concentrated at the edges of the lymphoid compartment or at the periphery of germinal centers. They were also numerous in the lamina propria of primary and secondary bronchial epithelia and in connective tissue septae in the lung parenchyma, but were conspicuously absent from air exchange regions of the lung. In germinal centers of 3-, 4-, and 6-week old chickens, there were large blast-like cells with weak cytoplasmic staining for all three immunoglobulins; in germinal centers of 6- and 8-week old chickens there were a few mature plasma cells, but no Ig+ blast-like cells. Cells with long cytoplasmic processes and diffuse cytoplasmic staining for IgG were in follicles of 3- and 4-week old chickens. These cells resembled follicular dendritic cells.

The numbers of IgPC in BALT and non-BALT regions are summarized in Table 1.

Figure 2a. BALT from 6-week old chicken stained for IgG. Positive cells (arrowheads) are below epithelium, in germinal center (G) and scattered among lymphocytes. Bronchial lumen (B). Bar = 200 μm.

Figure 2b. Non-BALT region from 6-week old chicken stained for IgA. Positive cells are in and below epithelium (arrows) and below muscularis mucosae (arrowheads). Bar = 100 μm.

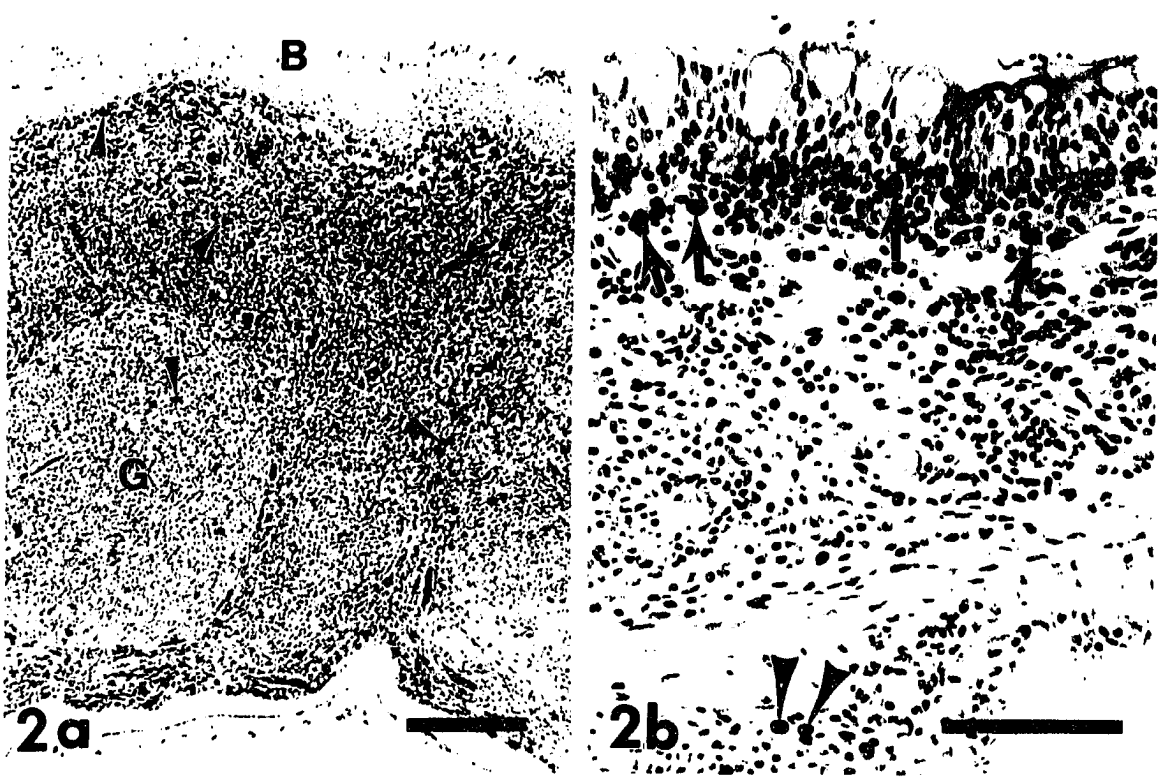

B
G
2a
2b

Table 1. Number of IgA-, IgM-, and IgG-producing cells in BALT and non-BALT regions.

Age	Number of cells per 1 mm^2 of tissue[a].					
	IgA		IgG		IgM	
	BALT	non-BALT	BALT	non-BALT	BALT	non-BALT
1 day	0	0	0	0	0	0
1 wk	0	0	8	36	0	0
2 wk	6	78	15	46	14	66
3 wk	19	58	43	51	59	195
4 wk	48	152	68	173	100	415
6 wk	55	160	267	323	222	268
8 wk	51	180	210	274	231	366

[a] Each number is the mean of 3 chickens.

The mean number of all IgPC, irrespective of region, increased with age ($P<0.0001$), with dramatic increases between 2 and 3 weeks of age for IgM, and between 3 and 4 weeks of age for IgG and IgA (Table 2). Overall, the number of cells producing IgM was greater than that of cells producing IgG, which was in turn greater than the number of cells producing IgA ($P<0.0001$).

When the mean numbers of IgPC in BALT and non-BALT regions were considered (Table 3), there were significantly more cells in non-BALT than in BALT regions at all ages ($P<0.003$).

Table 2. Number of IgA-, IgM- and IgG-producing cells[a].

	Age (in weeks)					
Isotype	2	3	4	6	8	Mean[b]
IgA	42	39	100	107	115	81
IgG	31	47	120	295	242	147
IgM	40	127	258	245	298	194
Mean[c]	**38**	**71**	**159**	**216**	**218**	

[a] Each number is the mean number of IgPC/mm^2 for 3 chickens.
[b] Mean number of IgPC for each isotype, standard error of the mean = 11, $P < 0.0001$
[c] Mean number of IgPC for each age, standard error of the mean = 18, $P < 0.0001$

T Lymphocytes CD4+ and CD8+ lymphocytes were distributed in distinctive patterns in BALT nodules. In chickens having nodules with mature germinal centers (3-8 week old birds), CD4+ cells were in tightly packed clusters forming large, asymmetric parafollicular caps around germinal centers (Fig 3a), whereas CD8+ cells were diffusely scattered throughout interfollicular areas of the nodules (Fig. 3b). There were a few CD4+ and CD8+ cells in germinal centers. In nodules without germinal centers, CD4+ cells formed single round clusters of cells. CD8+ cells were diffusely distributed as in nodules with germinal centers. Large numbers of CD4+ cells were associated with blood vessels in BALT; a few CD8+ cells were also seen there. In contrast to IgPC, CD4+ and CD8+ cells were scattered uniformly throughout

Table 3. Number of immunoglobulin-producing cells in BALT and non-BALT regions[a].

	Age (in weeks)					
Region	2	3	4	6	8	Mean[b]
BALT	12	40	72	181	164	94
non-BALT	63	101	247	250	273	187

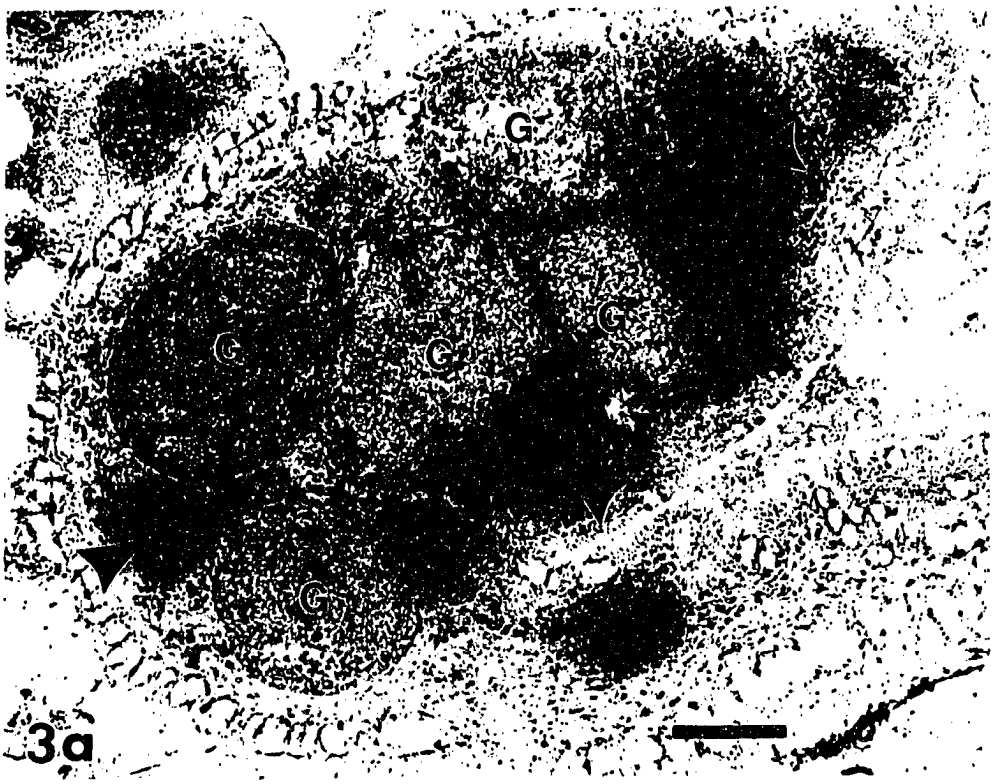
[a] Each number is the mean number of IgPC/mm^2 of 3 chickens.
[b] Mean number of IgPC for each region, standard error of the mean = 17, $P < 0.003$.

the lung parenchyma, both in connective tissue and air exchange regions. There were small clusters of CD4+ and CD8+ cells in the lung parenchyma. CD8+ clusters were seen in birds 1 week of age and older, while CD4+ clusters were found in birds 3 weeks of age and older.

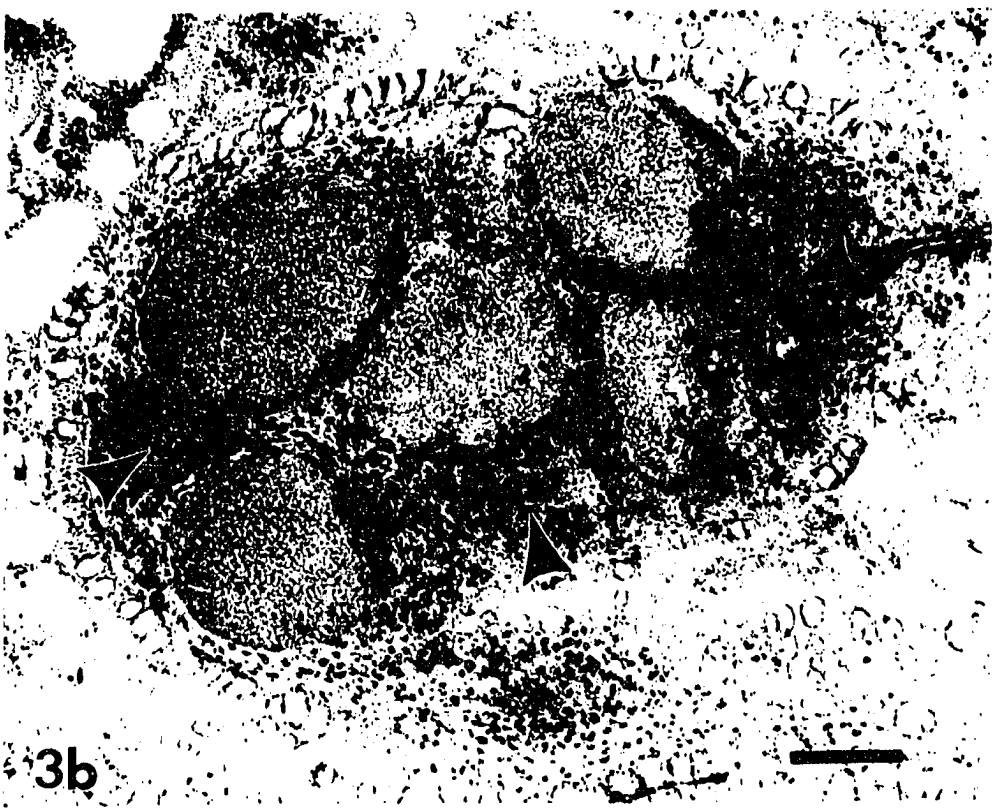
In sections from the three 1-day old chickens stained for T lymphocytes, there were no BALT nodules. However, a single small cluster of CD4+ and CD8+ cells was seen under the primary bronchus epithelium of one bird. In the other two birds examined, there were no CD4+ or CD8+ cells; however, there were CD3+ cells in small aggregates under the primary bronchus epithelium. At 1-week of age, there were clusters of both CD4+ and CD8+ cells in the primary bronchus epithelium.

Figure 3a. BALT nodule from 8-week old chicken stained for CD4. Arrowheads indicate masses of positive cells; germinal centers (G) are unstained. Bar = 200 μm.

Figure 3b. Section adjacent to that in previous figure stained for CD8. Positive cells (arrowheads) are scattered between germinal centers. Bar = 200 μm.



3a



3b

Intraepithelial lymphocytes in chickens at all ages examined were exclusively CD8+. These were usually located in the basal cell layer of the primary bronchial epithelium but were also occasionally seen in apical regions of the epithelium (Fig. 4).

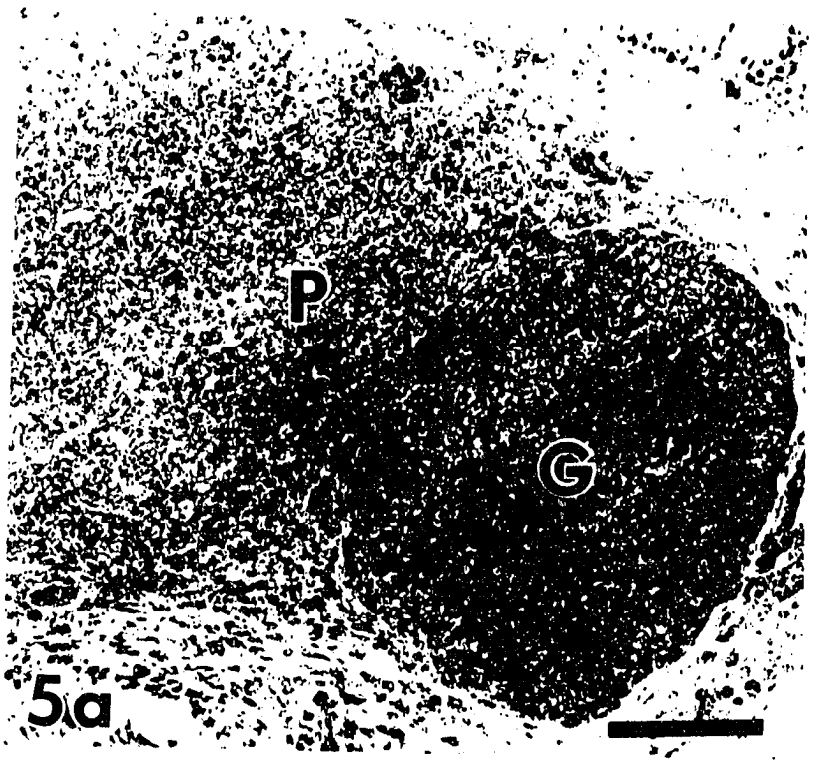
Non-plasmacytic B Lymphocytes In contrast to the findings for T lymphocytes, there were no CB3+ B lymphocytes associated with the primary bronchus epithelium until 2 weeks of age; however, there were CB3+ cells scattered throughout the lung parenchyma in 1-day and 1-week old chickens. In BALT from chickens 2 weeks of age and older, CB3+ cells were the predominant cell type in germinal centers and lymphoid nodules, and were scattered diffusely in the lamina propria and connective tissue of the lung parenchyma. They were numerous in the lymphoid regions around germinal centers where they often overlapped with CD4+ regions (Fig. 5a, 5b).

Figure 4. CD8-positive cells (arrowheads) in and under BALT epithelium from 8-week old chicken. Bar = 100 μm.

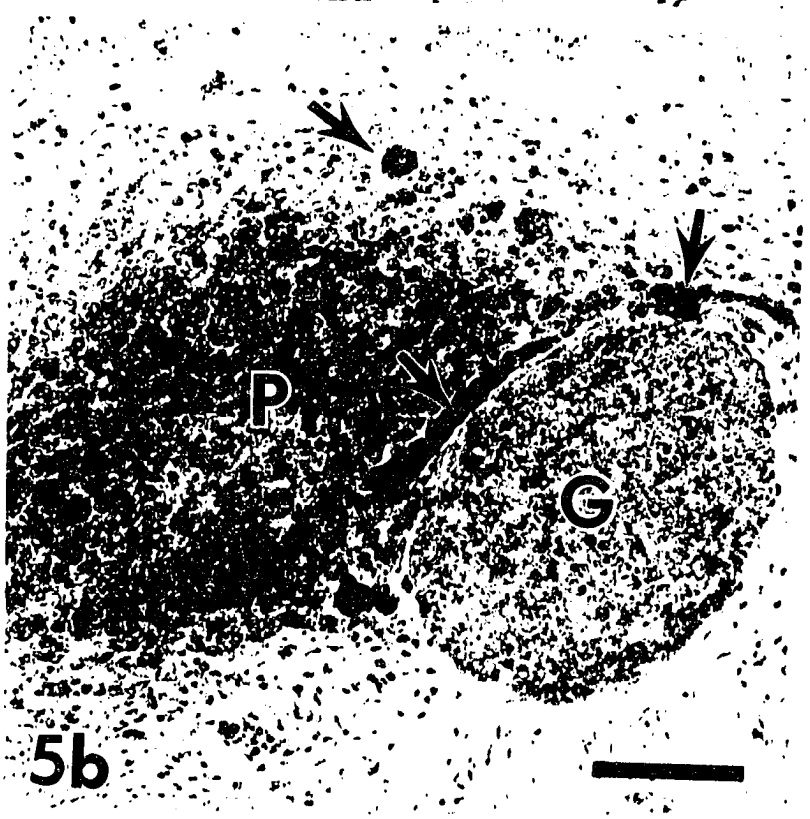

4

Figure 5a. BALT nodule from 8-week old chicken stained for CB3. Germinal center (G) and parafollicular region (P) are positive. Bar = 100 μm.

Figure 5b. Section adjacent to that in previous figure stained for CD4. Germinal center (G) is negative, while parafollicular region (P) is strongly positive. Large numbers of CD4-positive cells are associated with vessels (arrows). Bar = 100 μm.



5a



5b

DISCUSSION

In this study, the development and distribution of BALT nodules in SPF chickens followed the same time course as previously seen in BALT of conventional chickens (manuscript in preparation) and turkeys (4) and in lacrimal duct lymphoid tissue of conventional and SPF chickens (17). In all cases, lymphoid nodules were not fully developed before 2-3 weeks of age. Because this time course is the same in both SPF and conventional chickens, we believe BALT is a lymphoid tissue which is normally present in poultry but which may enlarge in response to respiratory pathogens (7). Birds lack a system of lymph nodes comparable to that in mammals, and mucosa-associated lymphoid tissues may functionally compensate for this deficiency by providing an environment similar to that in mammalian lymph nodes (18), designed to maximize effective interactions between antigen, antigen-presenting cells, and T and B lymphocytes.

The results of the immunocytochemical staining reported here confirm the presence in chicken BALT of lymphocyte populations necessary to initiate a humoral immune response; the full complement of T and B lymphocytes in BALT is in place by 2 weeks of age. The delay in lymphoid tissue development in chickens may be a consequence of delayed seeding of T lymphocytes from the thymus, which is not thought to begin until the fourth day after hatching (19). However, T lymphocytes are present in intestinal tissues of 3-day old

chicks (20), suggesting that they may leave the thymus earlier. In our study, we found T lymphocytes in all three of the 1-day old chicks examined, but in two of these chicks the T cells were immature as evidenced by the lack of CD4 or CD8 molecules. In contrast to T cells, emigration of B lymphocytes from the bursa begins before hatching and continues for about 3 weeks after hatching (21). This is consistent with our finding CB3+ cells in lung parenchyma of 1-day old chicks. The fact that B cells were not found in the epithelium until 2 weeks of age, a week later than the first T cells were seen, suggests that T cells may be involved in recruitment of B cells into the epithelium.

Mature plasma cells and B lymphocytes both were first seen in BALT at the same time, 2 weeks of age, thus the plasma cells in BALT at 1 and 2 weeks of age may have arisen as a result of antigen-priming in other tissues other than BALT. Subsequent generations of plasma cells could, however, arise via mucosal priming of the lymphocyte populations in BALT at 2 weeks of age and older.

The localizations of T and B lymphocytes in chicken BALT are generally similar to those in mammalian BALT (22-26), and are immunologically reasonable, assuming germinal centers in BALT function as do those in other secondary lymphoid tissues (27,28). CD4+ T helper cells were found in parafollicular caps around germinal centers, overlapping with CB3+ B lymphocytes. This localization would facilitate exposure of B lymphocytes to

cytokines produced by CD4+ T cells, thus providing for further proliferation and differentiation of B lymphocytes involved in humoral immune responses.

CD8+ T lymphocytes were more diffusely scattered in BALT nodules and were found more closely associated with epithelial cells than were CD4+ cells. This finding, is consistent with current knowledge of CD8+ cells, which are believed to have special functions in epithelia which besides cytotoxicity, include modulating expression of class II major histocompatibility proteins (29) and producing cytokines (30). In chicken intestinal epithelium, most CD8+ cells have a T cell receptor of the γ/δ type (20). It has been proposed that γ/δ T lymphocytes comprise a unique population of T cells that function primarily in surveillance of epithelial tissue, recognizing and destroying altered epithelial cells (31). Thus it seems likely that CD8+ cells in chicken BALT tissues, particularly if they are shown to carry the γ/δ T cell receptor, are important in monitoring the bronchial epithelium for pathogen-induced changes.

Our observation that lymphocytes around HEV are primarily T cells has also been reported for rat BALT (24). In mammals, lymphocyte recirculation is directed by receptor/ligand interactions that result in selective migration of lymphocytes into tissues (32,33). Whether this occurs in birds is not known, but the predominance of CD4+ cells associated with chicken HEV in BALT implies some degree of specific interaction.

We found IgG+ cells in 1-week old chickens and IgG+, IgA+, and IgM+ cells in chickens 2 weeks of age and older. Our findings are at odds with

some previous reports. For example, in a study using fluorescence microscopy, no IgPC were found in adult (5-month old) chicken BALT nodules (34). The technique we used (horseradish peroxidase-labeled streptavidin) is generally more sensitive than labeling with fluorescent antibodies. Differences in primary antibodies used may also explain the discrepancies. We do not believe the difference in age of chickens used is significant, since we have found large numbers of IgPC using immunocytochemistry in 1-year old chickens (unpublished data).

In a more recent study using frozen sections and monoclonal antibodies, IgM+ lymphocytes were seen in BALT of 5-day old SPF chickens, and IgM+ and IgG+ plasma cells were seen at 2 weeks of age (35). Again, differences in the procedures used may be responsible for the apparently conflicting results. Our results are more consistent with a recent study in which polyclonal sera and paraffin-embedded tissue sections were used (30). In this study IgG+ plasma cells were seen at 1 week of age and all three isotypes were seen at 2 weeks of age. Despite these minor conflicts in findings, we conclude that, due to a lack of plasma cells, for about the first week of life there is no local production of immunoglobulin in chicken BALT; and for the first 2 weeks there is no IgA. Thus protection against respiratory pathogens in birds for the first few weeks of life must be serum-derived, either from maternal sources or from production elsewhere in the bird.

The distribution of plasma cells at the periphery of chicken BALT nodules and in connective tissue suggests that, unlike some avian lymphoid tissues (such as those in the Harderian gland and Meckel's diverticulum), the primary function of BALT is not local production of immunoglobulin (17,36,37). The peripheral location of IgPC is similar to the localization in mammalian lymph nodes, where plasma cells are confined to medullary cords at some distance from germinal centers. Teleologically this arrangement is sensible, since free antibody downregulates antibody responses and would thus interfere with the activities in germinal centers (38).

The predominance of IgG+ cells in chicken BALT is consistent with other findings in chicken respiratory tract (39,40), Harderian gland (34) and salivary glands of birds vaccinated and challenged with Newcastle disease virus (37). Studies of Bordetella avium infection in turkeys also suggest an important role for IgG in avian respiratory immunity (41). Thus, while IgA may be of prime importance in mammalian mucosal systems (42), this seems not to be the case for avian systems.

In summary, our morphological studies show that in the primary bronchus of SPF chickens the full complement of lymphoid elements needed for a humoral immune response is lacking until about 2 weeks of age. After this time, populations of B lymphocytes, T helper and T cytotoxic/suppressor lymphocytes, and plasma cells are well developed. The spatial arrangement of the various lymphocyte populations in chicken BALT is similar to that found in

mammalian lymph nodes and suggests that a microenvironment specialized to facilitate the interaction between antigen and effector elements of the humoral immune response exists in avian BALT.

ACKNOWLEDGMENTS

We thank the following people for their assistance: Sarah Allmon for immunocytochemical procedures and cell counting; Elise Huffmann for immunocytochemical procedures and general technical assistance; Becky Smith for histological preparations; Margie Carter and Taka Miller for help with image analysis; Traci Koeppel for general technical assistance; and Lie-Ling Wu for help with statistical analyses.

REFERENCES

1. Brandtzaeg P: Overview of the mucosal immune system. *Curr Top Microbiol Immunol* 1989; 146:13-25.
2. Dahlgren U, Carlsson B, Jalili F, MacDonald R, Mascart-Lemone F, Nilsson K, Roberton D, Sennhauser F, Wold A, Hanson LA: Induction of the mucosal immune response. *Curr Top Microbiol Immunol* 1989; 146:155-160.
3. Bienenstock J, Johnston N, Perey DYE: Bronchial lymphoid tissue. I. Morphologic characteristics. *Lab Invest* 1973; 28:686-692.
4. Fagerland JA, Arp LH: A morphologic study of bronchus-associated lymphoid tissue in turkeys. *Am J Anat* 1990; 189:24-34.
5. Fedde MR: Anatomic and physiologic features of the avian respiratory system. *Proceedings of the 40th North Central Avian Disease Conference* 1989; pages 4-8.
6. Wang N, Banzett RB, Butler JP, Fredberg JJ: Bird lung models show that convective inertia effects inspiratory aerodynamic valving. *Respiration Physiology* 1988; 73:111-124.
7. van Alstine WG, Arp LH: Histologic evaluation of lung and bronchus-associated lymphoid tissue in young turkeys infected with Bordetella avium. *Am J Vet Res* 1988; 49:835-839.

8. Sato Y, Mukai K, Watanabe S, Goto M, Shimosato Y: The Amex method: a simplified technique of tissue processing and paraffin embedding with improved preservation of antigens for immunostaining. *J Pathol* 1986; 125:431-435.

9. McLean IW, Nakane PK: Periodate-lysine-paraformaldehyde fixative: A new fixative for immunoelectron microscopy. *J Histochem Cytochem* 1974; 22:1077-1083.

10. Hassell J, Hand AR: Tissue fixation with diimidoesters as an alternative to aldehydes. I. Comparison of cross-linking and ultrastructure obtained with dimethylsuberimidate and glutaraldehyde. *J Histochem Cytochem* 1974; 22:223-239.

11. Tokuyasu KT, Singer SJ: Improved procedures for immunoferritin labeling of ultrathin frozen sections. *J Cell Biol* 1976; 71:894-906.

12. Willingham MC, Yamada SS: Development of a new primary fixative for electron microscopic immunocytochemical localization of intracellular antigens in cultured cells. *J Histochem Cytochem* 1979; 27:947-960.

13. Pickel JM, Chen CH, Cooper MD: An avian B-lymphocyte protein associated with β 2-microglobulin. *Immunogenetics* 1990; 32:1-7.

14. Chan MM, Chen C-L H, Ager LL, Cooper MD: Identification of the avian homologues of mammalian CD4 and CD8 antigens. *J Immunol* 1988; 140:2133-2138.

15. Chen C-L H, Ager LL, Gartland GL, Cooper MD: Identification of a T3/T cell receptor complex in chickens. *J Exp Med* 1986; 164:375-380.

16. Bourne JA: *Handbook of Immunoperoxidase Staining Methods.* Carpenteria CA: DAKO Corporation. 1983.

17. Bang BG, Bang FB: Localized lymphoid tissues and plasma cells in paraocular and paranasal organ systems in chickens. *Am J Pathol* 1968; 53:735-751.

18. van Rooijen N: The "In situ" immune response in lymph nodes: a review. *Anat Rec* 1987; 218:359-364.

19. Seto F: Early development of the avian immune system. *Poult Sci* 1981; 60:1981-1995.

20. Bucy RP, Chen C-L H, Cihak J, Losch U, Cooper MD: Avian T cells expressing γ-Δ receptors localize in the splenic sinusoids and the intestinal epithelium. *J Immunol* 1988; 141:2200-2205.

21. Weill J-C, Reynaud C-A: The chicken B cell compartment. *Science* 1987; 238:1094-1098.

22. Simecka JW, Davis JK, Cassell GH: Distribution of Ia antigens and T lymphocyte subpopulations in rat lungs. *Immunol* 1986; 57:93-98.

23. Plesch BEC, Gamelkoorn GJ, van de Ende M: Development of bronchus associated lymphoid tissue (BALT) in the rat, with special

reference to T- and B-cells. *Dev Com Immunol* 1983; 7:179-188.21.

24. Otsuki Y, Ito Y, Magari S: Lymphocyte subpopulations in high endothelial venules and lymphatic capillaries of bronchus-associated lymphoid tissue in the rat. *Am J Anat* 1989; 184:139-146.

25. Crawford JM, Miller DA: Lymphocyte subpopulations in rat lungs and Peyer's patches. *Am Rev Resp Dis* 1984; 129:827-832.

26. Breel M, van der Ende M, Sminia T, Kraal G: Subpopulations of lymphoid and non-lymphoid cells in bronchus- associated lymphoid tissue (BALT) of the mouse. *Immunol* 1988; 63:657-662.

27. VanRooijen N: Antigen processing and presentation *in vivo*: the microenvironment as a crucial factor. *Immunol Today* 1990; 11:436-439.

28. Liu Y-J, Johnson GD, Fordon J, MacLennan ICM: Germinal centres in T-cell-dependent antibody responses. *Immunol Today* 1992; 13:17-21.

29. Cerf-Bensussan N, Quaroni A, Kurnick JT, Bhan AK: Intraepithelial lymphocytes modulate Ia expression by intestinal epithelial cells. *J Immunol* 1984; 132:2244-2252.

30. Taguchi T, Aicher WK, Fujihashi K, Yamamoto M, McGhee JR, Bluestone JA, Kiyono H: Novel function for intestinal intraepithelial lymphocytes:

Murine CD3+, γ/δ TCR+ T cells produce IFN-γ and IL-5. *J Immunol Meth* 1991; 147:3736-3744.

31. Janeway SA, Jr, Jones B, Hayday A: Specificity and function of T cells bearing γ δ receptors. *Immunol Today* 1988; 9:73-76.

32. Springer TA: The sensation and regulation of interactions with the extracellular environment: the cell biology of lymphocyte adhesion receptors. *Ann Rev Cell Biol* 1990; 6:359-402.

33. van der Brugge-Gamelkoorn GJ, Kraal G: The specificity of the high endothelial venule in bronchus- associated lymphoid tissue (BALT). *J Immunol* 1985; 134:3746-3750.

34. Bienenstock J, Gauldie J, Perey DYE: Synthesis of IgG, IgA, IgM by chicken tissues: immunofluorescent and C-14 amino acid incorporation studies. *J Immunol* 1973; 111:1112-1118.

35. Jeurissen SHM, Janse EM, Koch G, DeBoer GF: Postnatal development of mucosa-associated lymphoid tissues in chickens. *Cell Tissue Res* 1989; 258:119-124.

36. Eerola E, Veromaa T, Toivanen P: Special features in the structural organization of the avian lymphoid system. in: A Toivanen, P Toivanen, (eds) *Avian Immunology: Basis and Practice*, Vol. I, Boca Raton FL: CRC Press. 1987; pp 9-9.30.

37. Ewert DL, Barger BO, Eidson CS: Local antibody response in chickens: Analysis of antibody synthesis to Newcastle disease

virus by solid-phase radioimmunoassay and immunofluorescence with class-specific antibody for chicken immunoglobulins. *Infect Immun* 1979; 24:269-275.

38. Szakal AK, Kosco MH, Tew JG: Microanatomy of lymphoid tissue during humoral immune responses: structure function relationships. *Ann Rev Immunol* 1989; 7:91-109.

39. Arai N, Hashimoto Y, Kitagawa H, Kon Y, Kudo N: Immunohistochemical study on the distribution of lymphoid tissues in the upper alimentary and respiratory tracts of chickens. *Jpn J Vet Sci* 1988; 50:183-192.

40. Lawrence EC, Arnaud-Battandier F, Koski IR, Dooley NJ, Muchmore AV, Blaese RM: Tissue distribution of immunoglobulin-secreting cells in normal and IgA-deficient chickens. *J Immunol* 1979; 123:1767-1771.

41. Arp LH, Hellwig DH: Passive immunization versus adhesion of *Bordetella avium* to the tracheal mucosa of turkeys. *Avian Dis* 1988; 32:494-500.

42. Mestecky J, McGhee JR: Immunoglobulin A (IgA): Molecular and cellular interactions involved in IgA biosynthesis and immune response. *Adv Immunol* 1987; 40: 153-245.

PART IV

UPTAKE OF FERRITIN AND *BORDETELLA AVIUM* IN BRONCHUS-ASSOCIATED LYMPHOID TISSUE OF TURKEYS

Uptake of Ferritin and *Bordetella avium* in Bronchus-Associated Lymphoid Tissue of Turkeys

Jane A. Fagerland, Ronald K. Myers, and Lawrence H. Arp

Department of Veterinary Pathology

Iowa State University, Ames, Iowa 50011

ABSTRACT

In this study the ability of bronchus-associated lymphoid tissue (BALT) in turkeys to take up macromolecular and particulate materials, represented by ferritin and *Bordetella avium* respectively, was examined using transmission electron microscopy. Both live and ultraviolet-killed *B. avium* were tested. Uptake materials were instilled via intratracheal catheterization and allowed to remain in contact with the respiratory surfaces for 0, 10, 30, 60, and 90 minutes; uptake of bacteria was also examined after 120 minutes. Ferritin and *B. avium* were taken up by both ciliated and non-ciliated cells of BALT epithelium. Ferritin appeared in organelles associated with endocytosis (i.e., apical vesicles, endosomes, cytoplasmic vacuoles) and was apparently transported across epithelial cells, since it was also found in intercellular spaces. Bacteria appeared in the same cellular organelles as did ferritin, however they were not seen free in extracellular spaces. Macrophages containing bacteria were intimately associated with lymphocytes in lymphoid regions of BALT and within the epithelium; these were seen in turkeys instilled with either live or uv-killed bacteria. Thus, the epithelium overlying BALT in turkeys is able to take up both macromolecular and particulate materials. These materials are also accessible to macrophages, although whether they are taken up directly from the bronchial surface or whether they pass through epithelial

cells first could not be determined. This evidence supports a role for BALT in avian respiratory immune responses.

INTRODUCTION

Bronchus-associated lymphoid tissue (BALT) in poultry is a highly developed mucosa-associated lymphoid tissue thought to be important in generating protective mucosal immune responses (1,2). An initial requirement for the development of humoral responses is access of antigen to cells of the immune system; however, the normal function of mucosal epithelia is to create a barrier to foreign materials. These apparently conflicting functions are resolved by structural modifications in the epithelium of BALT that compromise the barrier. For example, there are reduced numbers of goblet cells in BALT epithelium of turkeys (2) and many other species (3-7). Also, ciliated cells are absent, reduced in number, or structurally altered over BALT lymphoid nodules in turkeys (2) and chickens (manuscript in preparation). In addition, non-ciliated epithelial cells of turkey BALT are attenuated to the extent that in some regions only a thin rim of cytoplasm separates materials in the bronchial lumen from underlying lymphocytes, heterophils, and macrophages (2).

In mammals (rabbits, rats, guinea pigs, calves, and miniature pigs), BALT epithelium has been shown to take up proteins including horseradish peroxidase and ferritin, as well as particulate materials such as mycobacteria, colloidal carbon, and latex beads (3,7-11). Whether similar uptake occurs in poultry has not been determined.

In the studies reported here, we tested the ability of BALT epithelium in 3-week old turkeys to take up a macromolecule, ferritin, and a bacterium, *Bordetella avium*. *B. avium* is a biologically relevant tracer organism; it is a non-invasive respiratory pathogen in turkeys and induces a humoral immune response detectable in respiratory secretions (12). Since live organisms often induce better mucosal immune responses than killed ones (13), we used both live and uv-killed *B. avium* to examine possible differences in uptake.

MATERIALS AND METHODS

Animals Three-week old unvaccinated turkeys (Willmar Poultry, Willmar MN) were used in these studies. They were obtained at 1 day of age, housed in a room containing no other animals, and given free access to food and water.

Treatments Turkeys were lightly anesthetized with 0.1 - 0.15 ml of an Acepromazine (10 mg/ml;Tech America, Elwood KS USA)/ketamine (100 mg/ml; Ft. Dodge Laboratories, Ft. Dodge IA USA) solution (1:4) and given 1 ml of tracer material via an 11 cm catheter inserted into the trachea through the mouth. Three materials were used to monitor uptake: ferritin, live *B. avium*, and ultraviolet (uv)-killed *B. avium*. Cationized ferritin (Sigma Chemical Co., St. Louis MO) was diluted to a concentration of 0.32 mg/ml in filter-sterilized 0.1 M phosphate-buffered saline, pH 7.2 (PBS). Live *B. avium* solutions were prepared as follows: A lawn of *B. avium* strain 75 was grown on brain-heart infusion agar (BHI). After 48 h incubation at 32°C, 5 ml of PBS were added to the BHI plate; the organisms were scraped from the surface, forced through a 21 gauge needle three times to break up clumps, and diluted to give an absorbance of 0.5 at 600 nm. At this concentration, there are $1x10^9$ colony-forming units of bacteria/ml. Uv-killed bacteria were prepared similarly, except that prior to instillation into trachea, an uncovered 10 cm Petri dish containing 5 ml of diluted culture was placed on a platform shaker 43 cm from a 15 watt uv lamp and exposed to uv irradiation for 20 minutes. Killing of organisms under

these conditions was ascertained by plating 50 μl of the suspension onto blood agar plates and incubating at 32°C for 48 hours.

Turkeys were killed at various time intervals after materials were instilled into tracheas. Birds receiving ferritin were killed immediately (0 minutes), and after 10, 30, 60, and 90 minutes of exposure; birds receiving bacteria were killed at 0, 10, 30, 60, 90, and 120 minutes after instillation. Three turkeys were used at each exposure time for a total of 51 experimental birds.

Tissue collection and processing Turkeys were killed with an overdose of sodium pentobarbital and immediately perfused intratracheally with 2.8% glutaraldehyde in 0.075 M Sorensen's phosphate buffer, pH 7.2. Tissues were prepared for transmission electron microscopy as previously described (2). Briefly, they were dehydrated in an ethanol series and embedded in epoxy resin. Thin sections from *B. avium*-exposed tissues were stained in 2% methanolic uranyl acetate (9 minutes) and Reynolds' lead citrate (9 minutes). Thin sections from ferritin-exposed lungs were stained for 60 seconds in a bismuth solution that selectively enhances the electron density of ferritin (14).

Controls A total of 16 control turkeys were used to determine the extent of morphological changes induced by the experimental procedure itself. One ml of PBS was instilled into tracheas and allowed to remain for the same time intervals as the tracers. Two PBS control turkeys were used at each exposure time in the ferritin study; 1 PBS control was used at each time in the *B. avium* studies. In addition, to determine anesthesia-induced morphologic

changes, two turkeys were anesthetized, allowed to recover for 5 minutes, then euthanatized. Lungs from the control birds were fixed and processed for electron microscopy as described above. To check for endogenous ferritin and non-specific staining, tissue sections from PBS controls were stained with bismuth and examined for ferritin-like particles.

RESULTS

Ferritin-uptake Ferritin appeared to be taken up by an endocytic process in BALT epithelium. After short exposure times (0-10 minutes), ferritin was attached to microvilli of both ciliated (CC) and non-ciliated (NCC) cells and was near vesicles in the plasma membrane (Fig. 1a, 1b). At 30 minutes, there was ferritin in apical vesicles, and in vacuoles at various levels within the cytoplasm of these cell types (Fig. 1c). By 60 minutes, it was seen between epithelial cells in the baso-lateral intercellular spaces (Fig. 1d). Ferritin was not seen in macrophages; however, it was difficult to detect in non-epithelial cells.

***Bordetella avium*-uptake** There were no differences between uptake of live and uv-killed bacteria. *B. avium* was taken up by both CC and NCC of BALT epithelium via a phagocytic process that appeared to be initiated by the wrapping of adjacent microvilli around bacteria (Fig. 2a, 2b). After 60, 90, and 120 minutes bacteria were seen in vacuoles at various levels (apical, intermediate, and basal) within epithelial cells (Fig. 3); however, they were not found free in intercellular spaces as was ferritin. At longer exposure times (120 minutes), large numbers of degenerate bacteria were in vacuoles of NCC (Fig. 4).

Live and uv-killed bacteria, were also found in intra-epithelial macrophages. In some cases, macrophages containing bacteria were in intimate physical contact with numerous lymphocytes (Fig 5a, 5b).

Figure 1a. Ferritin attached to glycocalyx on microvilli of non-ciliated cell after 30 minutes of exposure. Bar = 250 nm.

Figure 1b. Ferritin attached to microvilli of ciliated cell, near open vesicle, and in apical vesicle (arrow) after 60 minutes of exposure. Bar = 200 nm.

Figure 1c. Ferritin in cytoplasmic vacuole after 30 minutes of exposure. Bar = 300 nm.

Figure 1d. Ferritin in extracellular space after 60 minutes of exposure. Bar = 300 nm.

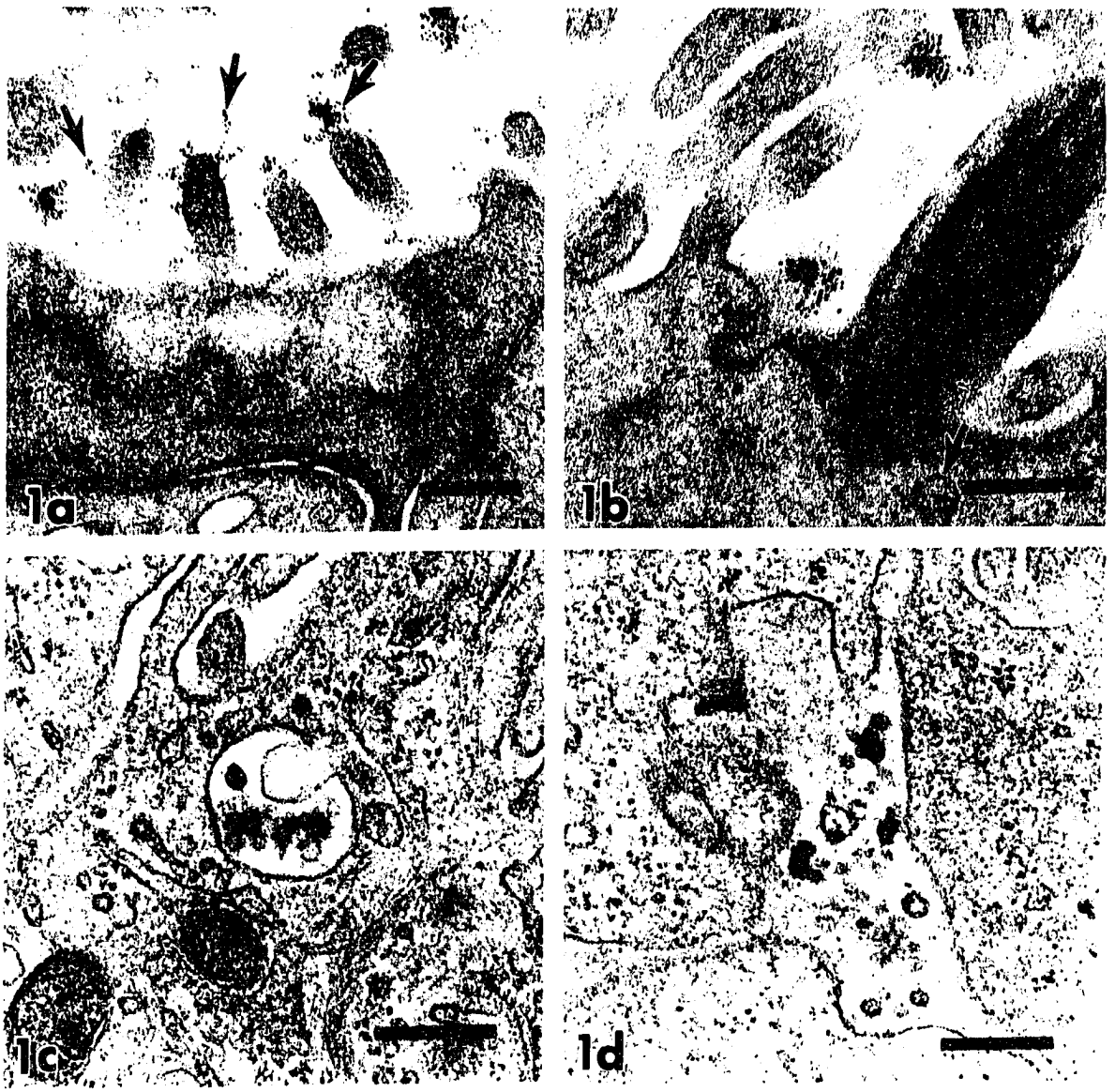

1a
1b
1c
1d

Figure 2a. Microvilli of ciliated cell around bacterium after 60 minutes of exposure to live *Bordetella avium*. Bar = 500 nm.

Figure 2b. Bacterium in apical cytoplasm of ciliated cell after apparent engulfment by microvilli. 60 minutes of exposure to live *Bordetella avium*. Bar = 500 nm.

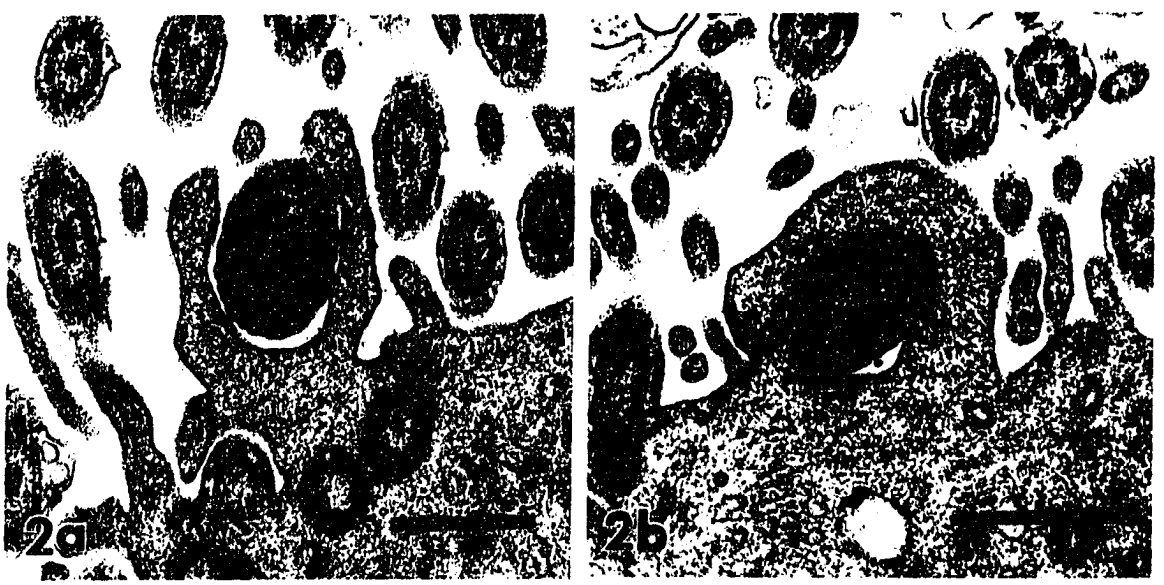

2a
2b

Figure 3. Bacterium in apical cytoplasm of non-ciliated cell after 120 minutes of exposure to live *Bordetella avium*. Bar = 500 nm.

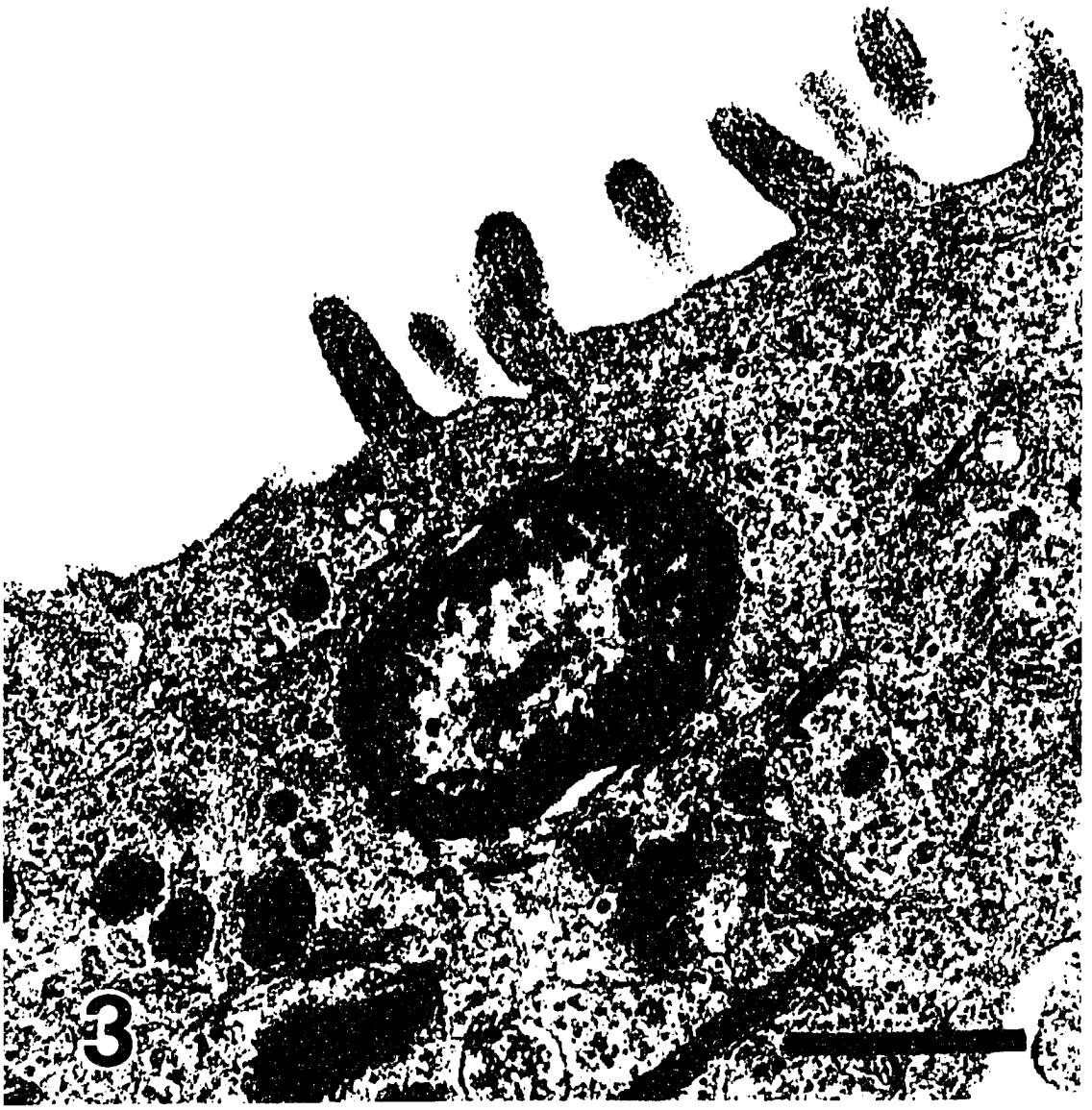

3

Figure 4. Bacteria in vacuoles of non-ciliated cells after 120 minutes of exposure to live *Bordetella avium*. Bar = 2 μm.

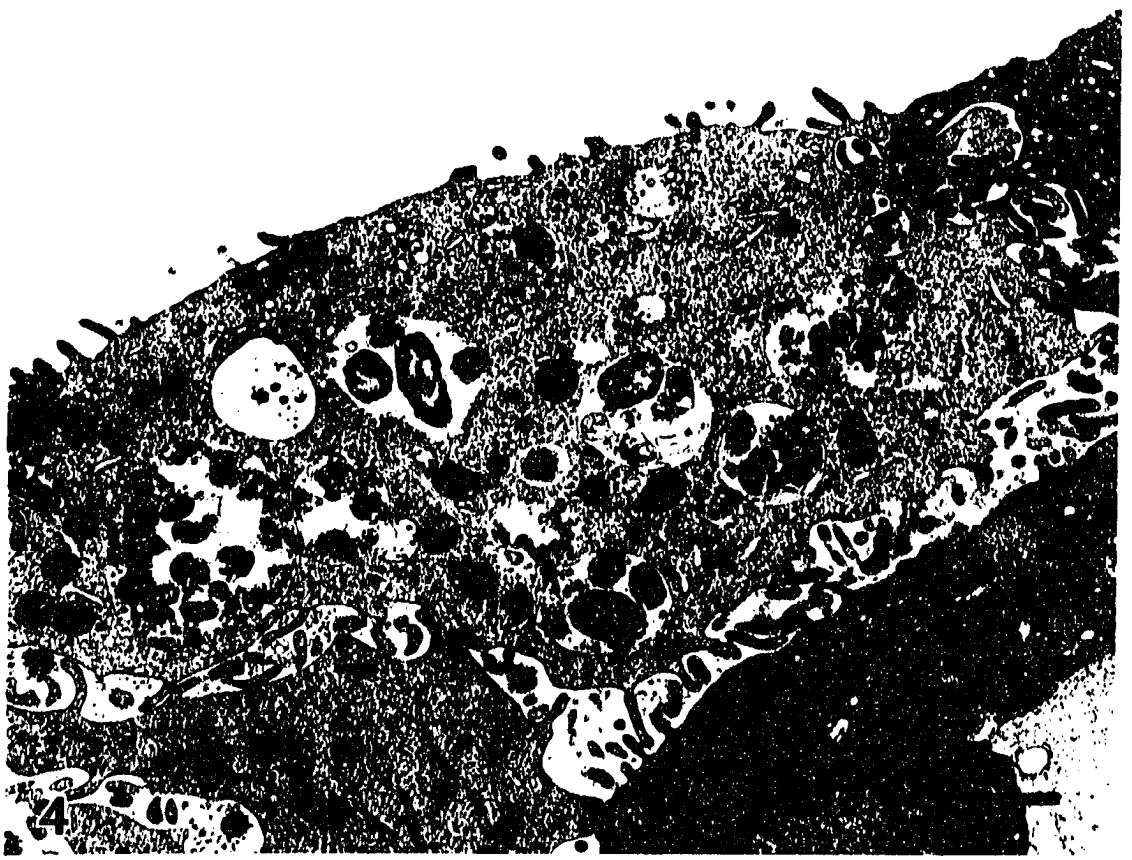
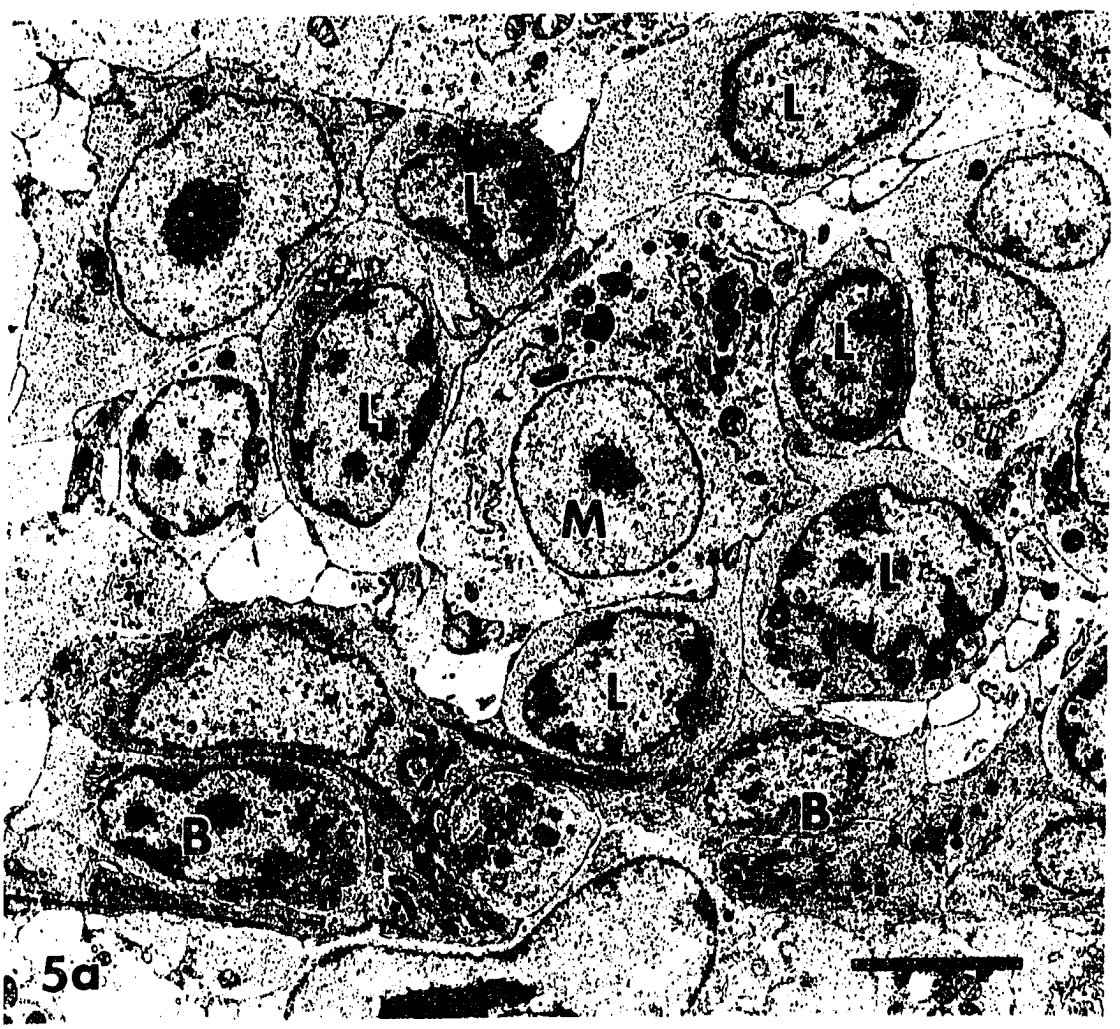
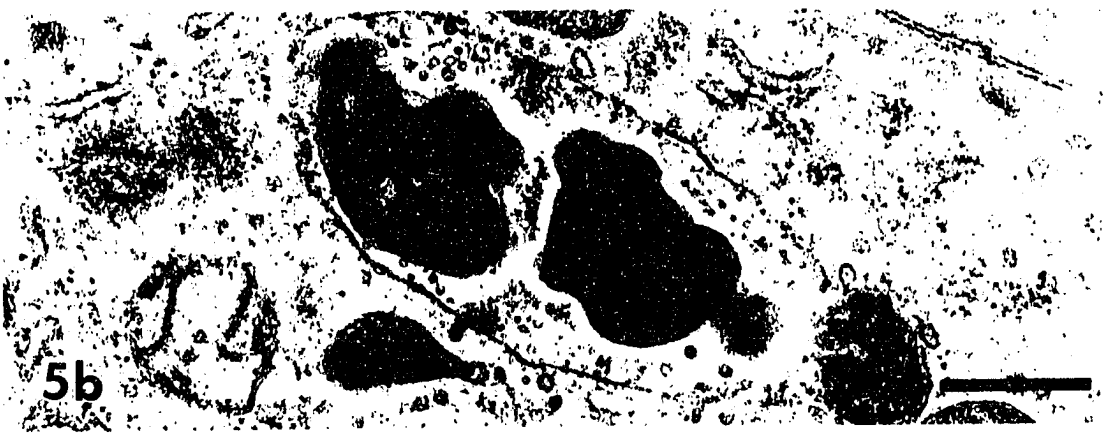

4

Figure 5a. Bacteria within intraepithelial macrophage (M) surrounded by lymphocytes (L) after 90 minutes exposure to uv-killed *Bordetella avium*. Basal cells of epithelium (B). Bar = 4 μm.

Figure 5b. Higher magnification of previous figure showing cell wall structure of degenerate bacteria in macrophage. Bar = 500 nm.



5a



5b

Controls In both experimental birds and controls, the ultrastructure of epithelial cells examined in this study was identical to that previously described for normal untreated turkeys (2). Treatment with the solutions of tracer materials did not result in increased numbers of apical vesicles or tubules in BALT epithelial cells. Surprisingly, no heterophil influx was stimulated in any of the turkeys by the procedures used. Neither ferritin nor bacteria were seen in any of the controls.

DISCUSSION

In this study, both NCC and CC of the BALT epithelium in 3-week old turkeys took up macromolecular and particulate materials, suggesting that BALT may be a route of uptake for antigenic materials in the respiratory tract. Macrophages in the epithelium also contained the tracer bacteria, although whether the bacteria were phagocytosed directly or relayed to macrophages from epithelial cells could not be determined. Epithelial cells containing bacteria were not in contact with lymphocytes, while macrophages were, hinting that macrophages may be the chief antigen-presenting cells here rather than epithelial cells. The ability of turkey BALT epithelial cells to present antigen has not been determined; however, in human bronchus and nasal epithelium, cells *not* associated with lymphoid tissues are effective antigen-presenting cells (15,16). Regardless of which cell in BALT actually presents antigen to lymphocytes, epithelial uptake of a non-invasive pathogen such as *B. avium* allows lymphocytes to "see" and respond to antigens which might otherwise remain undetected.

In mammals, there is much evidence to support the idea that antigen is transported in phagocytes to regional lymph nodes, where priming for respiratory immune responses occurs (17). Birds, however, have only a rudimentary system of lymphoid nodules that cannot function to the extent of their mammalian analogs (18). In avian species, well-developed lymphoid

nodules containing germinal centers exist in most parenchymal tissues (18); in addition, mucosa-associated lymphoid tissues in poultry are highly developed. BALT in chickens is more extensive than its mammalian counterpart (1) and has all of the lymphocyte populations needed to initiate humoral immune responses (manuscript in preparation). Thus in poultry, BALT may perform one of the major functions normally ascribed to mammalian lymph nodes, i.e., creating a microenvironment that facilitates interaction between antigen, antigen-presenting cells, and T and B lymphocytes (19).

Modified epithelial cells (M cells) overlying mucosal lymphoid nodules are thought to have similar structural and functional properties regardless of the mucosal tissue in which they occur (20). However, many generalizations about M cells do not fit BALT in general (4) and avian BALT in particular. In previous studies we have shown than, unlike M cells of laboratory mammals (21), cells in turkey BALT that otherwise resemble M cells by virtue of their intimate association with epithelial lymphocytes do not have apical vesicles, but do have a ruthenium red positive glycocalyx (2). Studies of rat intestinal M cells have indicated that materials are transported unchanged across the epithelial cells (22), while the work we describe here clearly shows degraded bacteria within epithelial cells. The fate of these degraded bacteria is not known, but there are several possibilities. Bacteria may be completely degraded, recycled into other materials within epithelial cells, and never exposed to the immune system. On the other hand, degraded fragments may associate with major

histocompatibility complex proteins and be expressed on epithelial cell surfaces in a form that can activate adjacent T lymphocytes. Alternatively, degraded materials may be exocytosed from epithelial cells, taken up by nearby B lymphocytes and macrophages for presentation to T lymphocytes. Unfortunately, the reagents and cell systems to test these possibilities are not currently available in turkeys.

ACKNOWLEDGMENTS

The authors wish to thank Elise Huffman and Traci Koeppel for excellent technical assistance.

REFERENCES

1. Bienenstock J, Johnston N, Perey DYE: Bronchial lymphoid tissue. I. Morphologic characteristics. *Lab Invest* 1973; 28:686-692.

2. Fagerland JA, Arp LH: A morphologic study of bronchus-associated lymphoid tissue in turkeys. *Am J Anat* 1990; 189:24-34.

3. Fournier M, Vai F, Derenne JP, Pariente R: Bronchial lymphoepithelial nodules in the rat: Morphologic features and uptake and transport of exogenous proteins. *Amer Rev Resp Dis* 1977; 116:685-694.

4. Sminia T, van der Brugge-Gamelkoorn GJ: Structure and function of bronchus-associated lymphoid tissue (BALT). *CRC Crit Rev Immunol* 1989; 9:119-150.

5. van der Brugge-Gamelkoorn GJ, van de Ende M, Sminia T: Changes occurring in the epithelium covering the bronchus-associated lymphoid tissue of rats after intratracheal challenge with horseradish peroxidase. *Cell Tiss Res* 1986; 245:439-444.

6. Bienenstock J, Johnston N: A morphologic study of rabbit bronchial lymphoid aggregates and lymphoepithelium. *Lab Invest* 1976; 35:343-348.

7. Myrvik QN, Racz P, Racz KT: Ultrastructural studies on bronchial-associated lymphoid tissue (BALT) and lymphoepithelium

in pulmonary cell- mediated reactions in the rabbit. in: MR Escobar, H Friedman, (eds) *Macrophages and Lymphocytes: Nature, Functions, and Interaction*, New York: Plenum Press. 1980; pp 145-153.

8. Tenner-Racz K, Racz P, Myrvik QN, Ockers JR, Geister R: Uptake and transport of horseradish peroxidase by lymphoepithelium of the bronchus-associated lymphoid tissue in normal and Bacillus Calmette-Guerin-immunized and challenged rabbits. *Lab Invest* 1979; 41:106-115.

9. van der Brugge-Gamelkoorn G, van de Ende M, Sminia T: Uptake of antigens and inert particles by bronchus associated lymphoid tissue (BALT) epithelium in the rat. *Cell Biol Int Rep* 1985; 9:524.

10. Bienenstock J, Johnston N, Perey DYE: Bronchial lymphoid tissue II. Functional characteristics. *Lab Invest* 1973; 28:693-698.

11. Heilmann P, Muller G: Aufnahme intratracheal verabreichter Tuschepartikeln durch das bronchusassoziierte lymphatische Gewebe bei Kalb und Ferkel. *Arch Exper Vet Med Leipzig* 1987; 41:242-248.

12. Hellwig DH, Arp LH: Identification of *Bordetella avium* antigens recognized after experimental inoculation in turkeys. *J Am Vet Med Assoc* 1990; 51:1188-1191.

13. Bergmann K, Waldman RH: Stimulation of secretory antibody following oral administration of antigen. *Rev Infect Dis* 1988; 10:939-950.

14. Ainsworth SK, Karnovsky MJ: An ultrastructural staining method for enhancing the size and electron opacity of ferritin in thisn sections. *J Histochem Cytochem* 1972; 20:225-229.

15. Mezzetti M, Sosloperto M, Fasoli A, Mattoli S: Human bronchial epithelial cells modulate CD3 and mitogen-induced DNA synthesis in T cells but function poorly as antigen-presenting cells compared to pulmonary macrophages. *J Allergy Clin Immunol* 1991; 87:930-938.

16. Kalb TH, Chuang MT, Marom Z, Mayer L: Evidence for accessory cell function by class II MHC antigen-expressing airway epithelial cells. *Am J Respir Cell Mol Biol* 1991; 4:320-329.

17. Bice DE, Shopp GM: Antibody responses after lung immunization. *Exp Lung Res* 1988; 14:133-155.

18. Payne LN: The lymphoid system. in: DJ Bell, BM Freeman, (eds): *Physiology and Biochemistry of the Domestic Fowl*, New York: Academic Press. 1971; pp 985-1037.

19. VanRooijen N: Antigen processing and presentation *in vivo*: the microenvironment as a crucial factor. *Immunol Today* 1990; 11:436-439.

20. Wolf JL, Bye WA: The membranous epithelial (M) cell and the mucosal immune system. *Ann Rev Med* 1984; 35:95-112.

21. Owen RL, Pierce NF, Apple RT, Cray WC: M cell transport of *Vibrio cholerae* from the intestinal lumen into Peyer's patches: a mechanism for antigen sampling and for microbial transepithelial migration. *J Infect Dis* 1986; 153:1108-1118.

22. Owen RL, Apple RT, Bhalla DK: Morphometric and cytochemical analysis of lysosomes in rat Peyer's patch follicle epithelium: Their reduction in volume fraction and acid phosphatase content in M cells compared to adjacent enterocytes. *Anat Rec* 1986; 216:521-527.

GENERAL SUMMARY AND DISCUSSION

The work described here characterizes the structure and function of bronchus-associated lymphoid tissue (BALT) in poultry. The first study used light and electron microscopy to examine the development of BALT in turkeys. Its purpose was to determine whether the structure of turkey BALT was similar to that of BALT in other species and whether the cellular elements needed to initiate immune responses against respiratory pathogens were present in turkey BALT. The second paper is a brief description of the structural development of BALT in conventional chickens; it was a prelude to the work described in the third manuscript, an immunocytochemical study of the changing populations of immunoglobulin-producing cells and T and B lymphocytes in BALT from specific pathogen free chickens 1 day through 8 weeks of age. In the fourth study, transmission electron microscopy was used to document the ability of BALT epithelium to sample antigens from the bronchial lumen. Tracer materials (ferritin and *Bordetella avium*) were intratracheally instilled into 3-week old turkeys. BALT nodules were examined after several exposure times to determine the location of tracers and the cell types involved in uptake.

The results of morphological studies of turkey and chicken BALT were similar. The anatomical location of BALT in chickens and turkeys is the same: it occurs at points where secondary bronchi branch from the primary bronchus. In both chickens and turkeys, BALT undergoes similar age-related changes.

Nodules are larger and more numerous in older birds, and the relationship of lymphocyte and epithelial compartments is altered with age. In 1- to 3-week old birds, the epithelium is extensively infiltrated with lymphocytes, forming a *lymphoepithelium*. Epithelial cells in these younger birds are altered, being primarily non-ciliated and squamous or cuboidal, rather than ciliated and columnar. Goblet cells are sparse in the lymphoepithelium. These changes probably compromise the mucociliary barrier over lymphoid nodules in younger poultry. In older birds (4 weeks of age and up), lymphocyte and epithelial cell compartments are isolated from each other by increasing amounts of connective tissue. There are few intraepithelial lymphocytes compared to younger birds. The lymphoid compartment appears to be quiescent, with fewer mitotic lymphocytes in germinal centers and smaller numbers of lymphocytes overall.

Epithelia that line mucosal surfaces are generally structured to prevent contact with invasive organisms and toxic materials. However, it would be advantageous for an organism to have a means of sampling potential pathogens from the external environment and priming lymphocytes for subsequent exposures. The morphological features of poultry BALT appear to be designed to facilitate antigen sampling and lymphocyte priming. The anatomical location is one in which turbulence in airflow patterns would favor deposition of materials on epithelial surfaces and the surfaces themselves are altered to enhance contact with foreign materials.

The time course of BALT development in poultry also suggests that its function is to generate a population of primed lymphocytes (and probably memory cells, as well). The greatest activity in the lymphocyte compartment of avian BALT occurs during the first 2-4 weeks of life, the same amount of time required for a humoral response to develop. Presumably, a bird would be exposed to most of the antigens in its normal environment, including a few potential pathogens, during the first few weeks of life. These exposures would initiate immune responses, producing a protective reserve of immunocompetent cells ready to expand when re-exposed to the same antigens.

The ages at which T lymphocytes, B lymphocytes, and plasma cells first appear in chicken BALT support a role for BALT in generating antigen-primed lymphocytes. T lymphocyte subsets and non-immunoglobulin-producing B lymphocytes are in BALT before plasma cells appear in large numbers and could be involved in the priming process that precedes B cell differentiation into plasma cells. Plasma cells are deficient in young birds (for about the first two weeks of life), then begin to increase in numbers, making exponential jumps at 3 or 4 weeks of age. The numbers of plasma cells plateau at 6 weeks of age, suggesting that the chickens have been exposed to most novel antigens by this age and have generated specific antibody-producing cells.

Several conclusions can be drawn from the relative numbers of plasma cells in BALT and non-BALT regions of the bronchus. There are significantly fewer immunoglobulin-producing cells in BALT regions compared to non-BALT

regions, supporting the idea that BALT is primarily involved in generating immune responses, but not in local production of antibody. In mammalian mucosal systems, IgA is considered to be the immunoglobulin-isotype of primary importance; but in the studies described here, IgG- and IgM-producing cells far outnumber IgA-producers. Thus, while IgA may still have an important role in mucosal immunity in poultry, the roles of IgG and IgM may be substantial as well.

The immunocytochemical studies confirmed the presence in avian BALT of lymphocyte populations necessary to initiate and regulate immune responses. T helper and cytotoxic lymphocyte subsets are present and localized in areas consistent with their known functions in immune responses. For example, T helper cells are concentrated near germinal centers, where they overlap B lymphocyte clusters. This localization places T helper cells where their cytokines can easily reach B lymphocytes, directing their proliferation and differentiation into plasma cells. Cytotoxic T cells are scattered throughout lymphoid nodules, but were found adjacent to and within the bronchial epithelium. This localization, too, is consistent with their known function in surveillance of epithelial cells for presence of foreign antigens. B lymphocytes, as would be expected, are the predominant cell type in germinal centers, and also overlap T helper cells in parafollicular regions.

The final studies in this research document the ability of both ciliated and non-ciliated cells of BALT to take up macromolecular and particulate materials.

In the ultrastructural studies of poultry BALT, no specializations (such as apical vesicles and tubules) for extensive uptake of materials were seen. However, both ferritin and *Bordetella avium* are taken up by significant numbers of cells in BALT. Ferritin appears in vesicles and small vacuoles characteristic of an endocytic process; the bacteria appear to be taken up by phagocytosis. Both of these materials are taken up in the absence of an inflammatory response, suggesting that uptake can occur in normal bronchi. Macrophages also take up bacteria, but whether they are taken up from the lumen or relayed by epithelial cells could not be determined.

From the results of this research, the following scenario is proposed for the generation of immune responses to bronchial antigens. Foreign materials, both macromolecular and particulate, are taken up by BALT epithelium and perhaps macrophages. These materials are processed and presented to T lymphocytes in BALT nodules. Several cell types are available in poultry BALT for antigen processing and presentation: epithelial cells, macrophages, B lymphocytes, and dendritic cells. Localizations of lymphocytes ensure interactions between cytokines from T helper cells and B lymphocytes. Further selection and differentiation of B cells occurs within germinal centers, such that B cells exiting from germinal centers are programmed either to differentiate into plasma cells or to enter a resting phase as memory cells.

Many of the elements of this scenario are currently untestable in birds due to lack of the reagents and cell lines that have facilitated work in

mammalian systems. However, several interesting questions that could be addressed remain. For example, it would be useful to determine whether uptake of materials across BALT epithelium is physiologically relevant. This could be done by using tracer studies similar to those described here, but combining them with techniques to identify antigen-specific plasma cells. Double labelling immunocytochemical techniques or ELISPOT methods could be used to identify plasma cells secreting antibody to a known antigen. This would show whether uptake across BALT actually initiates a specific immune response, or whether it is simply a means of removing foreign materials from the bronchial lumen.

A second question of interest involves the fate of antigens taken up across BALT epithelia. In mammals, antigens from the lungs are carried to regional lymph nodes in phagocytes and respiratory immune responses are generated in lymph nodes rather than in mucosal lymphoid tissues. Since birds lack competent lymph nodes, it would be useful to determine whether antigens cross the BALT epithelium and remain in underlying lymphoid tissues, or if they are taken up by macrophages and heterophils and transported to other secondary lymphoid organs such as spleen or bone marrow. Studies of this type in mammals have used large tracers such as colloidal carbon, latex beads, and xenogeneic erythrocytes and could be done in poultry.

The morphological studies in this research suggest that in birds older than 8 weeks of age, lymphoid elements of BALT are relatively inactive. It would be

interesting to determine whether these tissues could be induced to form lymphoepithelia in response to new antigens. This could be easily determined by intratracheally exposing older chickens and turkeys to a novel antigen and looking for morphological changes in BALT tissues with light microscopy.

The ultimate goal of this research was to provide basic information to facilitate the development of effective vaccines against respiratory pathogens in poultry. These studies have confirmed the ability of BALT epithelium to take up materials from the bronchial lumen and have documented that the cells necessary to induce immune responses are present in the respiratory tract of chickens and turkeys older than 2 weeks of age. They suggest that aerosol vaccines might be effective in 2- to 4-week old birds, but might not be protective in younger birds that have not yet developed the immune machinery to respond to bronchial antigens. In these birds, maternal antibody or perhaps parenteral vaccination to generate serum antibodies might provide the best protection.

ADDITIONAL LITERATURE CITED

1. Abu-Ghazaleh RI, Fujisawa T, Mestecky J, Kyle RA, Gleich GJ: IgA-Induced eosinophil degranulation. *J Immunol.* 1989; 142:2393-2400.

2. Aitken JD, Parry SH: Local immunity in the respiratory tract of the chicken. I. Transudation of circulating antibody in normal and virus-infected birds. *Immunol.* 1976; 31:33-37.

3. Alexander DJ: Newcastle disease and other paramyxovirus infections. in: BW Calnek, (ed) *Diseases of Poultry*, Iowa: Iowa State University, Ames. 1991; pp 496-519.

4. Ambrosius H, Hadge D: Chicken immunoglobulins. *Vet Immunol Immunopathol.* 1987; 17:57-67.

5. Anderson ML, Moore PF, Hyde DM, Dungworth DL: Bronchus associated lymphoid tissue in the lungs of cattle: relationship to age. *Res Vet Sci.* 1986; 41:211-220.

6. Apodaca G, Bomsel M, Arden J, Breitfeld PP, Tang K, Mostov KE: The polymeric immunoglobulin receptor: A model protein to study transcytosis. *J Clin Invest.* 1991; 87:1877-1882.

7. Arai N, Hashimoto Y, Kitagawa H, Kon Y, Kudo N: Immunohistochemical study on the distribution of lymphoid tissues

in the upper alimentary and respiratory tracts of chickens. *Jpn J Vet Sci.* 1988; 50:183-192.

8. Arp LH, Hellwig DH: Passive immunization versus adhesion of *Bordetella avium* to the tracheal mucosa of turkeys. *Avian Dis.* 1988; 32:494-500.

9. Arp LH, Skeeles JK: Bordetellosis (turkey coryza). in: BW Calnek, (ed) *Diseases of Poultry*, Iowa: Iowa State University Press, Ames. 1991; pp 277-288.

10. Baba T, Ando T, Nukina M: Effect of bursectomy and thymectomy on *Pasteurella multocida* infection in chickens. *J Med Microbiol.* 1978; 11:281-288.

11. Baba T, Masumoto K, Nishida S, Kajikawa T, Mitsui M: Harderian gland dependency of immunoglobulin A production in the lacrimal fluid of chicken. *Immunol.* 1988; 65:67-71.

12. Bang BG, Bang FB: Localized lymphoid tissues and plasma cells in paraocular and paranasal organ systems in chickens. *Am J Pathol.* 1968; 53:735-751.

13. Befus AD, Johnston N, Leslie GA, Bienenstock J: Gut-associated lymphoid tissue in the chicken: I. Morphology, ontogeny, and some functional characteristics of Peyer's patches. *J Immunol.* 1980; 125:2626-2632.

14. Bencina D, Svetlin A, Dorrer D, Tadina-Jaksic T: Humoral and local antibodies in chickens with mixed infection with three *Mycoplasma* species. *Avian Pathology*. 1991; 20:325-334.

15. Benedict AA, Berestecky JM: Special features of avian immunoglobulins. in: A Toivanen, P Toivanen, (eds) *Avian Immunology: Basis and Practice*, Florida: CRC Press, Inc, Boca Raton. 1987; pp 114-125.

16. Bergmann K, Waldman RH: Stimulation of secretory antibody following oral administration of antigen. *Rev Infect Dis*. 1988; 10:939-950.

17. Bessen D, Fischetti VA: Synthetic peptide vaccine against mucosal colonization by group A streptococci I. Protection against a heterologous M serotype with shared C repeat region epitopes. *J Immunol*. 1990; 145:1251-1256.

18. Bice DE, Degen MA, Harris DL, Muggenburg BA: Recruitment of antibody-forming cells in the lung after local immunization is nonspecific. *Am Rev Resp Dis*. 1982; 126:635-639.

19. Bice DE, Shopp GM: Antibody responses after lung immunization. *Exp Lung Res*. 1988; 14:133-155.

20. Bienenstock J: Bronchus-associated lymphoid tissue. in: J Bienenstock, (ed) *Immunology of the Lung and Upper Respiratory Tract*, New York: McGraw-Hill Book Company. 1984; pp 96-118.

21. Bienenstock J, Befus AD: Mucosal immunology. *Immunol.* 1980; 41:249-269.

22. Bienenstock J, Johnston N: A morphologic study of rabbit bronchial lymphoid aggregates and lymphoepithelium. *Lab Invest.* 1976; 35:343-348.

23. Bienenstock J, Johnston N, Perey DYE: Bronchial lymphoid tissue. I. Morphologic characteristics. *Lab Invest.* 1973; 28:686-692.

24. Bienenstock J, Johnston N, Perey DYE: Bronchial lymphoid tissue II. Functional characteristics. *Lab Invest.* 1973; 28:693-698.

25. Bland PW, Warren LG: Antigen presentation by epithelial cells of the rat small intestine I. Kinetics, antigen specificity and blocking by anti-Ia antisera. *Immunology.* 1986; 58:1-7.

26. Blanton RA, Kupper TS, McDougall JK, Dower S: Regulation of interleukin 1 and its receptor in human keratinocytes. *Proc Natl Acad Sci USA.* 1989; 86:1273-1277.

27. Blythman HE, White RG: Effect of early bursectomy on germinal centre and immunoglobulin production in chickens. *Immunol.* 1977; 33:671-677.

28. Brain RD: Free cells in the lungs: some aspects of their role, quantitation, and regulation. *Archives of Internal Medicine.* 1970; 126:477-487.

29. Breel M, van der Ende M, Sminia T, Kraal G: Subpopulations of lymphoid and non-lymphoid cells in bronchus- associated lymphoid tissue (BALT) of the mouse. *Immunol.* 1988; 63:657-662.

30. Brune K, Leffell MS, Spitznagel JK: Microbicidal activity of peroxidaseless chicken heterophile leukocytes. *Infect Immun.* 1972; 5:283-287.

31. Brune K, Spitznagel JK: Peroxidaseless chicken leukocytes: isolation and characterization of antibacterial granules. *J Infect Dis.* 1973; 127:84-94.

32. Butcher EC: Leukocyte-endothelial cell recognition: Three (or more) steps to specificity and diversity. *Cell.* 1991; 67:1033-1036.

33. Calnek BW: Immunologically rational vaccination programs. *Proceedings of the International Union of Immunological Societies.* 1983; 66:177-180.

34. Castro GA: Immunological regulation of epithelial function. *Am J Physiol.* 1982; 243:G321-329.

35. Cebra JJ, Fuhrman JA, Griffin P, Rose FV, Schweitzer PA, Zimmerman D: Changes in specific B cells and the dissemination of the primed state in vivo following antigenic stimulation by different mucosal routes. *Acta Anat.* 1984; 53:541-549.

36. Cebra JJ, Gearhart PJ, Halsey JF, Hurwitz JL, Shahin RB: Role of environmental antigens in the ontogeny of the secretory immune response. *J Reticulo Soc*. 1980; 28:61s-71s.

37. Chamberlain DW, Nopajaroonsri C, Simon GT: Ultrastructure of pulmonary lymphoid tissue. *Am Rev Resp Dis*. 1973; 108:621-631.

38. Chhabra PC, Goel MC: Normal profile of immunoglobulins in sera and tracheal washings of chickens. *Res Vet Sci*. 1980; 29:148-152.

39. Childers NK, Bruce MG, McGhee JR: Molecular mechanisms of immunoglobulin A defense. *Ann Rev Microbiol*. 1989; 43:503-536.

40. Chute HL, Richard JL: Fungal infections. in: BW Calnek, (ed) *Diseases of Poultry*, Iowa: Iowa State University Press, Ames. 1991; pp 326-334.

41. Clancy R, Bienenstock J: A framework for immunization strategy. in: J Bienenstock, (ed) *Immunology of the Lung and Upper Respiratory Tract*, New York: McGraw-Hill Book Company. 1984; pp 216-231.

42. Cornes JS: Number, size, and distribution of Peyer's patches in the human small intestine. *Gut*. 1965; 6:225-233.

43. Crawford JM, Miller DA: Lymphocyte subpopulations in rat lungs and Peyer's patches. *Am Rev Resp Dis*. 1984; 129:827-832.

44. Darbyshire JH: Immunity to avian infectious bronchitis virus. in: ME Rose, LN Payne, BM Freeman, (eds) *Avian Immunology*, Edinburgh: British Poultry Science Ltd. 1981; pp 205-226.

45. Diamond G, Zasloff M, Eck H, Brasseur M, Maloy WL, Bevins CL: Tracheal antimicrobial peptide, a cystein-rich peptide from mammalian tracheal mucosa: Peptide isolation and cloning of a cDNA. *Proc Natl Acad Sci USA*. 1991; 88:3952-3956.

46. Duijvestijn A, Hamann A: Mechanisms and regulation of lymphocyte migration. *Immunol Today*. 1989; 10:23-28.

47. Duncan RL, McArthur WP: Partial characterization and the distribution of chicken mononuclear cells bearing the Fc receptor. *J Immunol*. 1978; 120:1014-1020.

48. Easterday BC: Immunity to Newcastle disease and avian influenza. in: ME Rose, LN Payne, BM Freeman, (eds) *Avian Immunology*, Edinburgh: British Poultry Science Ltd. 1981; pp 179-185.

49. Easterday BC, Hinshaw VS: Influenza. in: BW Calnek, (ed) *Diseases of Poultry*, Iowa: Iowa State University Press, Ames. 1991; pp 532-551.

50. Eerola E, Veromaa T, Toivanen P: Special features in the structural organization of the avian lymphoid system. in: A Toivanen, P Toivanen, (eds) *Avian Immunology: Basis and Practice*, Vol. I, Boca Raton FL: CRC Press. 1987; pp 9-9.

51. Ewert DL, Eidson CS: Effect of bursectomy and depletion of immunoglobulin A on antibody production and resistance to respiratory challenge after local or systemic vaccination of chickens with Newcastle disease virus. *Infect Immun*. 1977; 18:146-150.

52. Fahey KJ, York JJ: Cytotoxic activity of avian lymphoid cells. in: A Toivanen, P Toivanen, (eds) *Avian Immunology: Basis and Practice*, Vol. 1, Boca Raton, FL: CRC Press. 1987; pp 179-193.

53. Faulk WP, McCormick JN, Goodman JR, Yoffey JM, Fudenberg HH: Peyer's patches: Morphologic studies. *Cell Immunol*. 1971; 1:500-520.

54. Ficken MD, Edwards JF, Lay JC: Induction, collection, and partial characterization of induced respiratory macrophages of the turkey. *Avian Dis*. 1986; 30:766-771.

55. Fix AS, Arp LH: Morphologic characterizaiton of conjunctiva-associated lymphoid tissue in chickens. *Am J Vet Res*. 1991; 52:1852-1859.

56. Fournier M, Vai F, Derenne JP, Pariente R: Bronchial lymphoepithelial nodules in the rat: Morphologic features and uptake and transport of exogenous proteins. *Amer Rev Resp Dis*. 1977; 116:685-694.

57. Fredericksen TL, Sharma JM: Purification of avian T cell growth factor and immune interferon using gel filtration high resolution

chromatography. in: WT Weber, DL Ewert, (eds) *Avian Immunology*, New York: Alan R Liss, Inc. 1987; pp 145-156.

58. Fulton RM, Reed WM, DeNicola DB: Light microscopic and ultrastructural characterization of cells recovered by respiratory-tract lavage of 2- and 6-week-old chickens. *Avian Dis.* 1990; 34:87-98.

59. Gadaleanu V: Ovalbumin-induced immunomorphologic reaction of rat lung with emphasis on bronchus-associated lymphoid tissue (BALT). *Rev Rou Morphol Embryol Physiol.* 1982; 28:65-69.

60. Geppert TD, Lipsky PE: Antigen presentation by cells that are not of bone marrow origin. *Reg Immunol.* 1989; 2:60-71.

61. Gillette KG: Local antibody response in avian infectious bronchitis: Virus-neutralizing antibody in tracheobronchial secretions. *Avian Dis.* 1981; 25:431-443.

62. Gough RE, Alexander DJ: Comparison of duration of immunity in chickens infected with a live infectious bronchitis vaccine by three different routes. *Res Vet Sci.* 1979; 26:329-332.

63. Gray JG, Roberts JF, Dillman RC, Simmons DG: Pathogenesis of change in the upper respiratory tracts of turkeys experimentally infected with an *Alcaligenes faecalis* isolate. *Infect Immun.* 1983; 42:350-355.

64. Gregson RL, Davey MJ, Prentice DE: Postnatal development of bronchus-associated lymphoid tissue (BALT) in the rat, *Rattus norvegicus*. *Lab Anim*. 1979; 13:231-238.

65. Gregson RL, Davey MJ, Prentice DE: Bronchus-associated lymphoid tissue (BALT) in the laboratory-bred and wild rat, *Rattus norvegicus*. *Lab Anim*. 1979; 13:239-243.

66. Grey HM, Sette A, Buus S: How T cells see antigen. *Sci Am*. 1989; 261:56-64.

67. Grimes JE, Wyrick PB: Chlamydiosis (ornithosis). in: BW Calnek, (ed) *Diseases of Poultry*, Iowa: Iowa State University Press, Ames. 1991; pp 311-325.

68. Gross WB: Factors affecting the development of respiratory disease complex in chickens. *Avian Dis*. 1990; 34:607-610.

69. Gross WB: Colibacillosis. in: BW Calnek, (ed) *Diseases of Poultry*, Iowa: Iowa State University Press, Ames. 1991; pp 138-144.

70. Guy JS, Barnes HJ, Smith L: Increased virulence of modified-live infectious larygotracheitis vaccine virus following bird-to-bird passage. *Avian Dis*. 1991; 35:348-355.

71. Hadge D, Ambrosius H: Comparative studies on the structure of biliary immunoglobulins of some avian species. II. Antigenic properties of the biliary immunglobulins of chicken, turkey, duck and goose. *Dev Comp Immunol*. 1988; 12:319-329.

72. Hall JG, Hopkins J, Orlans E: Studies on the lymphocytes of sheep. III. Destination of lymph-borne immunoblasts in relation to their tissue of origin. *Eur J Immunol.* 1977; 7:30-37.

73. Hanson LE, Bagust TJ: Laryngotracheitis. in: BW Calnek, (ed) *Diseases of Poultry*, Iowa: Iowa State University, Ames. 1991; pp 496-519.

74. Hayter RB, Besch EL: Airborne-particle deposition in the respiratory tract of chickens. *Poult Sci.* 1974; 53:1507-1511.

75. Heilmann P, Muiler G: Aufnahme intratracheal verabreichter Tuschepartikeln durch das bronchusassoziierte lymphatische Gewebe bei Kalb und Ferkel. *Arch Exper Vet Med Leipzig.* 1987; 41:242-248.

76. Hillam RP, Bice DE, Muggenburg BA: Lung localization of antibody-forming cells stimulated in distant peripheral lymph nodes. *Immunol.* 1985; 55:257-261.

77. Hinshaw VS, Sheerar MG, Larsen D: Specific antibody responses and generation of antigenic variants in chicken immunized against a virulent avian influenza virus. *Avian Dis.* 1990; 34:80-86.

78. Holmes HC: Neutralizing antibody in nasal secretions of chickens following administration of avian infectious bronchitis virus. *Archiv fur die gesamte virusforschung.* 1973; 43:235-241.

79. Hopkins SR, Yoder HW, Jr: Reversion to virulence of chicken-passaged infectious bronchitis vaccine virus. *Avian Dis.* 1986; 30:221-223.

80. Hoshi H, Mori T: Inhibition of the germinal center formation in the spleen by x-irradiations of the thymus in chickens. *Tohoku J Exp Med.* 1973; 109:97-111.

81. Jeurissen SHM, Janse EM, Koch G, DeBoer GF: Postnatal development of mucosa-associated lymphoid tissues in chickens. *Cell Tissue Res.* 1989; 258:119-124.

82. Joel DD, Chanana AD: Comparison of pulmonary and intestinal lymphocyte migrational patterns in sheep. *Ann N Y Acad Sci.* 1985; 459:56-66.

83. Jordan FTW: Immunity to infectious laryngotracheitis. in: ME Rose, LN Payne, BM Freeman, (eds) *Avian Immunology,* Edinburgh: British Poultry Science Ltd. 1981; pp 245-261.

84. Julius MH: Cellular interactions involved in T-dependent B-cell activation. *Immunol Today.* 1982; 3:295-299.

85. Kaiserlian D, Vidal K, Revillard J-P: Murine enterocytes can present soluble antigen to specific class II-restricted CD4+ T cells. *Eur J Immunol.* 1989; 19:1513-1516.

86. Kalb TH, Chuang MT, Marom Z, Mayer L: Evidence for accessory cell function by class II MHC antigen-expressing airway epithelial cells. *Am J Respir Cell Mol Biol.* 1991; 4:320-329.

87. Kaltreider HB: Local immunity. in: J Bienenstock, (ed) *Immunology of the Lung and Upper Respiratory Tract,* New York: McGraw-Hill Book Company. 1984; pp 191-215.

88. Keren DF, McDonald RA, Carey JL: Combined parenteral and oral immunization results in an enhanced mucosal immunoglobulin A response to *Shigella flexneri. Infect Immun.* 1988; 56:910-915.

89. King KJ, Cavanaugh D: Infectious bronchitis. in: BW Calnek, (ed) *Diseases of Poultry,* Iowa: Iowa State University Press, Ames. 1991; pp 471-484.

90. Lawrence EC, Arnaud-Battandier F, Koski IR, Dooley NJ, Muchmore AV, Blaese RM: Tissue distribution of immunoglobulin-secreting cells in normal and IgA-deficient chickens. *J Immunol.* 1979; 123:1767-1771.

91. Lebacq-Verheyden A, Vaerman J, Heremans JF: Quantification and distribution of chicken immunoglobulins IgA, IgM and IgG in serum and secretions. *Immunol.* 1974; 27:683-692.

92. Levenson VI, Chernokhvostova EV, Lyubinskaya MM, Salamatova SA, Dzhikidze EK, Stasilevitch CK: Parenteral immunization with *Shigella* ribosomal vaccine elicits local IgA response and primes for

mucosal memory. *Inflammation Int Arch Allergy Appl Immunol.* 1988; 87:25-31.

93. Lillehoj HS: Intestinal intraepithelial and splenic natural killer cell responses to eimerian infections in inbred chickens. *Infect Immun.* 1989; 57:1879-1884.

94. Lillehoj HS: Lymphocytes involved in cell-mediated responses and methods to assess cell-mediated immunity. *Poult Sci.* 1991; 70:1154-1164.

95. Liu Y-J, Johnson GD, Fordon J, MacLennan ICM: Germinal centres in T-cell-dependent antibody responses. *Immunol Today.* 1992; 13:17-21.

96. Mair TS, Batten EH, Stokes CR, Bourne FJ: The histological features of the immune system of the equine respiratory tract. *J Comp Pathol.* 1987; 97:575-586.

97. Mandi Y, Garssadi SI, Beladi I: Comparison of roles of serine esterase in chicken and human natural cytotoxicity. *Dev Comp Immunol.* 1990; 14:113-119.

98. Mandi Y, Seprenyi G, Pusztal R, Beladi I: Are granulocytes the main effector cells of natural cytotoxicity in chickens? *Immunobiol.* 1985; 170:284-292.

99. Mayrhofer G, Pugh CW, Barclay AN: The distribution, ontogeny and origin in the rat of Ia-positive cells with dendritic morphology and

of Ia antigen in epithelia, with special reference to the intestine. *Eur J Immunol.* 1983; 13:112-122.

100. Mazanec MB, Nedrud JG, Liang X, Lamm ME: Transport of serum IgA into murine respiratory secretions and its implications for immunization strategies. *J Immunol.* 1989; 142:4275-4281.

101. McCorkle FM, Stinson RS, Olah I, Glick B: The chickens's femoral-lymph nodules: T and B cells and the immune response. *J Immunol.* 1979; 123:667-669.

102. McDermott MR, Befus AD, Bienenstock J: The structural basis for immunity in the respiratory tract. *Int Rev Exp Pathol.* 1982; 23:47-99.

103. Mestecky J, McGhee JR: Immunoglobulin A (IgA): Molecular and cellular interactions involved in IgA biosynthesis and immune response. *Adv Immunol.* 1987; 40:153-245.

104. Mezzetti M, Sosloperto M, Fasoli A, Mattoli S: Human bronchial epithelial cells modulate CD3 and mitogen-induced DNA synthesis in T cells but function poorly as antigen-presenting cells compared to pulmonary macrophages. *J Allergy Clin Immunol.* 1991; 87:930-938.

105. Moriya O, Ischikawa Y: Age-related changes of J-chain positive cells in chickens. *Acta Histochem.* 1991; 90:103-108.

106. Mueller AP, Sato K, Glick B: The chicken lacrimal gland, gland of Harder, caecal tonsil, and accessory spleens as sources of antibody-producing cells. *Cell Immunol.* 1971; 2:140-152.

107. Myers CD: Role of B cell antigen processing and presentation in the humoral immune response. *FASEB J.* 1991; 5:2547-2553.

108. Myers RK, Arp LH: Pulmonary clearnace and lesions of lung and air sac in passively immunized and unimmunized turkeys following exposure to aerosolized *Escherichia cili. Avian Dis.* 1987; 31:622-628.

109. Mygind N, Winther B: Immunological barriers in the nose and paranasal sinuses. *Acta Otolaryngol (Stockh).* 1987; 103:363-368.

110. Myrvik QN, Racz P, Racz KT: Ultrastructural studies on bronchial-associated lymphoid tissue (BALT) and lymphoepithelium in pulmonary cell- mediated reactions in the rabbit. in: MR Escobar, H Friedman, (eds) *Macrophages and Lymphocytes: Nature, Functions, and Interaction*, New York: Plenum Press. 1980; pp 145-153.

111. Nagy ZA, Feher G: Histometric analysis of the chicken spleen during primary immune response to soluble bovine serum albumin. I. Relationship between quantitative changes of thymus-type and bursa-type splenic lymphocytes and splenomegaly. *A Immun-Forsch.* 1972; 143:223-244.

112. Nagy ZA, Horvath E, Urban Z: Cytological changes and antigen capture in chicken spleen during the secondary immune response to homologous and cross-reacting antigens. *Z Immun-Forsch.* 1975; 150:1-23.

113. Nakache M, Berg EL, Streeter PR, Butcher EC: The mucosal vascular addressin is a tissue-specific endothelial cell adhesion molecule for circulating lymphocytes. *Nature.* 1989; 337:179-181.

114. Nash DR, Holle B: Local and systemic cellular immune responses in guinea-pigs given antigen parenterally or directly into the lower respiratory tract. *Clin Exp Immunol.* 1973; 13:573-583.

115. Ochs DL, Toth TE, Pyle RH, Siegel PB: Cellular defense of the avian respiratory system: effects of *Pasteurella multocida* on respiratory burst activity of avian respiratory tract phagocytes. *Am J Vet Res.* 1988; 49:2081-2084.

116. Ogra PL, Fishaut M, Gallagher MR: Viral vaccination via the mucosal routes. *Rev Infect Dis.* 1980; 2:352-369.

117. Olah I, Glick B, Taylor RL: Meckel's diverticulum. II. A novel lymphopithelial organ in the chicken. *The Anat Rec.* 1984; 208:253-263.

118. Otsuki K, Nakamura T, Kawaoka Y, Tsubokura M: Interferon induction by several strains of avian infectious bronchitis virus, a coronavirus, in chickens. *Acta virol.* 1988; 32:55-59.

119. Otsuki Y, Ito Y, Magari S: Lymphocyte subpopulations in high endothelial venules and lymphatic capillaries of bronchus-associated lymphoid tissue in the rat. *Am J Anat.* 1989; 184:139-146.

120. Owen RL: Sequential uptake of horseradish peroxidase by lymphoid follicle epithelium of Peyer's patches in the normal unobstructed mouse intestine: an ultrastructural study. *Gastroenterology.* 1977; 72:440-451.

121. Pabst R: The anatomical basis for the immune function of the gut. *Anat Embryol.* 1987; 176:135-144.

122. Page LA: Studies on immunity to chlamydiosis in birds, with particular reference to turkeys. *Am J Vet Res.* 1975; 36:597-600.

123. Page LA: Stimulation of cell-mediated immunity to chlamydiosis in turkeys by inoculation of chlamydial bacterin. *Am J Vet Res.* 1978; 39:473-480.

124. Pankow W, von Wichert P: M cell in the immune system of the lung. *Respiration.* 1988; 54:209-219.

125. Parr EL, Parr MB, Thapar M: A comparison of specific antibody responses in mouse vaginal fluid after immunization by several routes. *Journal of Reproductive Immunology.* 1988; 14:165-176.

126. Parry SH, Aitken ID: Local immunity in the respiratory tract of the chicken. II. The secretory immune response to Newcastle disease virus and the role of IgA. *Vet Microbiol.* 1977; 2:143-165.

127. Payne LN: The lymphoid system. in: DJ Bell, BM Freeman, (eds) *Physiology and Biochemistry of the Domestic Fowl*, New York: Academic Press. 1971; pp 985-1037.

128. Plesch BEC, Gamelkoorn GJ, van de Ende M: Development of bronchus associated lymphoid tissue (BALT) in the rat, with special reference to T- and B-cells. *Dev Comp Immunol.* 1983; 7:179-188.

129. Pober JS, Cotran RS: Immunologic interactions of T lymphocytes with vascular endothelium. *Adv Immunol.* 1991; 50:261-302.

130. Powell PC: Immune mechanisms in infections of poultry. *Vet Immunol Immunopathol.* 1987; 15:87-113.

131. Powell PC: Macrophages and other nonlymphoid cells contributing to immunity. in: A Toivanen, P Toivanen, (eds) *Avian Immunology: Basis and Practice*, Vol. I, Boca Raton FL: CRC Press. 1987; pp 195-207.

132. Pusztai R, Tarodi B, Beladi I: Production and characterization of interferon induced in chicken leukocytes by concanavalin A. *Acta Virol.* 1986; 30:131-136.

133. Racz P, Tenner-Racz K, Myrvik QN, Fainter LK: Functional architecture of bronchial associated lymphoid tissue and

lymphoepithelium in pulmonary cell-mediated reactions in the rabbit. *J Reticulo Soc*. 1977; 22:59-83.

134. Rennard SI, Beckman J, Daughton D, Ertl RF, Koyama S, Rickard K, Romberger D, Rubenstein I, Shoji S, Sisson J, Spurzen J, Thompson AB, von Essen S, Robbins RA: The role of airway epithelium in cellular migration. in: SG Farmer, DWP Hay, (eds) *The Airway Epithelium: Physiology, Pathophysiology, and Pharmacology*, New York: Marcel Dekker, Inc. 1991.

135. Reynolds HY: Immunoglobulin G and its function in the human respiratory tract. *Mayo Clin Proc*. 1988; 63:161-174.

136. Reynolds HY: Immunologic system in the respiratory tract. *Physiol Rev*. 1991; 71:1117-1133.

137. Rhoades KR, Rimler RB: Pasteurellosis. in: BW Calnek, (ed) *Diseases of Poultry*, Ames, Iowa: Iowa State University Press. 1991; pp 145-162.

138. Richardson J, Bouchard T, Ferguson CC: Uptake and transport of exogenous proteins by respiratory epithelium. *Lab Invest*. 1976; 35:307-314.

139. Ritter MA, Rozing J, Schuurman H: The true function of the thymus? *Immunol Today*. 1988; 9:189-193.

140. Robbins RA, Shoji S, Linder J, Gossman GL, Allington LA, Klassen LW, Rennard SI: Bronchial epithelial cells release chemotactic activity for lymphocytes. *Am J Physiol.* 1989; 257:L109-L115.

141. Roitt IM, Brostoff J, Male DK: *Immunology*. New York: Harper & Row. 1989; 101-102.

142. Rose FV, Cebra JJ: Isotype commitment of B cells and dissemination of the primed state after mucosal stimulation with *Mycoplasma pulmonis*. *Infect Immun*. 1985; 49:428-434.

143. Roy MJ, Ruiz A, Varvayanis M: A novel antigen is common to the dome epithelium of gut- and bronchus-associated lymphoid tissues. *Cell Tissue Res*. 1987; 248:635-644.

144. Russell MW, Mestecky J: Induction of the mucosal immune response. *Rev Infect Dis*. 1988; 10 :S440-440s446.

145. Russell MW, Reinholdt J, Kilian M: Anti-inflammatory activity of human IgA antibodies and their Fab- (alpha) fragments: inhibition of IgG-mediated complement activation. *Eur J Immunol*. 1989; 19:2243-2249.

146. Saif YM, Moorhead PD, Dearth RN, Jackwood DJ: Observations on *Alcaligenes faecalis* infection in turkeys. *Avian Dis*. 1980; 24:665-684.

147. Schat KA, Calnek BW, Weinstock D: Cultured avian lymphocytes with natural killer cell activity. *Prog Clin Biol Res*. 1987; 238:157-169.

148. Sertl K, Takemura T, Tschachler E, Ferrans VJ, Kaliner MA, Shevach EM: Dendritic cells with antigen-presenting capability reside in airway epithelium, lung parenchyma, and visceral pleura. *J Exp Med*. 1986; 163:436-451.

149. Sharma JM, Tizard I: Avian cellular immune effector mechanisms - a review. *Avian Pathology*. 1984; 13:357-376.

150. Simecka JW, Davis JK, Cassell GH: Distribution of Ia antigens and T lymphocyte subpopulations in rat lungs. *Immunol*. 1986; 57:93-98.

151. Sminia T, van der Brugge-Gamelkoorn GJ: Structure and function of bronchus-associated lymphoid tissue (BALT). *CRC Critical Reviews in Immunology*. 1989; 9:119-150.

152. Sneller MC, Strober W: M cells and host defense. *J Infect Dis*. 1986; 154:737-741.

153. Spencer J, Gyure LA, Hall JG: IgA antibodies in the bile of rats. III. The role of intrathoracis lymph nodes and the migration pattern of their blast cells. *I*. 1983; 48:687-693.

154. Stearns RC, Barnas GM, Walski M, Brain JD: Phagocytosis in the gas exchange region of avian lung. *Fed Proc*. 1986; 45:959.

155. Stearns RC, Barnas GM, Walski M, Brain JD: Deposition and phagocytosis of inhaled particles in the gas exchange region of the duck, *Anas platyrhynchos*. *Respir Physiol.* 1987; 67:23-36.

156. Stein-Streilein J, Guffee J, Fan W: Locally and systemically derived natural killer cells participate in defense against intranasally inoculated influenza virus. *Reg Immunol.* 1988; 1:100-105.

157. Tenner-Racz K, Racz P, Myrvik QN, Ockers JR, Geister R: Uptake and transport of horseradish peroxidase by lymphoepithelium of the bronchus-associated lymphoid tissue in normal and Bacillus Calmette-Guerin-immunized and challenged rabbits. *Lab Invest.* 1979; 41:106-115.

158. Thapar MA, Parr EL, Parr MB: Secretory immune responses in mouse vaginal fluid after pelvic, parenteral or vaginal immunization. *Immunol.* 1990; 70:121-125.

159. Thorbecke GJ, Gordon HA, Wostman B, Wagner M, Reyniers JA: Lymphoid tissue and serum gamma globulin in young germfree chickens. *J Infect Dis.* 1957; 101:237-251.

160. Thunold S, Schauenstein K, Wolf H, Thunold KS, Wick G: Localization of IgGFc and complement receptors in chicken lymphoid tissue. *Scandinav J Immunol.* 1981; 14:145-152.

161. Toivanen A, Toivanen P: Histocompatibility requirements for cellular cooperation in the chicken: Generation of germinal centers. *J Immunol.* 1977; 118:431-436.

162. Toth TE, Pyle RH, Caceci T, Siegel PB, Ochs D: Cellular defense of the avian respiratory system: influx and nonopsonic phagocytosis by respiratory phagocytes activated by *Pasteurella multocida*. *Infect Immun.* 1988; 56:1171-1179.

163. Toth TE, Siegel P, Veit H: Cellular defense of the avian respiratory system. Influx of phagocytes: elicitation versus activation. *Avian Dis.* 1987; 31:861-867.

164. Toth TE, Siegel PB: Cellular defense of the avian respiratory tract: paucity of free-residing macrophages in the normal chicken. *Avian Dis.* 1986; 30:67-75.

165. Toth TE, Veit H, Gross WB, Siegel PB: Cellular defense of the avian respiratory system: protection against *Escherichia coli* airsacculitis by *Pasteurella multocida*-activated respiratory phagocytes. *Avian Dis.* 1988; 32:681-687.

166. Vainio O, Toivanen A: Cellular cooperation in immunity. in: A Toivanen, P Toivanen, (eds) *Avian Immunology: Basis and Practice*, Vol. II, Boca Raton FL: CRC Press. 1987; pp 1-12.

167. van Alstine WG, Arp LH: Histologic evaluation of lung and bronchus-associated lymphoid tissue in young turkeys infected with *Bordetella avium*. *Am J Vet Res*. 1988; 49:835-839.

168. van der Brugge-Gamelkoorn G, van de Ende M, Sminia T: Uptake of antigens and inert particles by bronchus associated lymphoid tissue (BALT) epithelium in the rat. *Cell Biol Int Rep*. 1985; 9:524.

169. van der Brugge-Gamelkoorn GJ, Dijkstra CD, Sminia T: Characterization of pulmonary macrophages and bronchus-associated lymphoid tissue (BALT) macrophages in the rat. An enzyme-cytochemical and immunocytochemical study. *Immunobiol*. 1985; 169:553-562.

170. van der Brugge-Gamelkoorn GJ, Kraal G: The specificity of the high endothelial venule in bronchus- associated lymphoid tissue (BALT). *J Immunol*. 1985; 134:3746-3750.

171. van der Brugge-Gamelkoorn GJ, Plesch BEC, Sminia T, Langevoort HL: Specific antibody-forming cells in bronchus-associated lymphoid tissue (BALT) and lung of the rat after intratracheal challenge with horseradish peroxidase. *Virchows Arch (Cell Pathol)*. 1985; 49:269-276.

172. van der Brugge-Gamelkoorn GJ, van de Ende M, Sminia T: Changes occurring in the epithelium covering the bronchus- associated

lymphoid tissue of rats after intratracheal challenge with horseradish peroxidase. *Cell Tissue Res.* 1986; 245:439-444.

173. van der Brugge-Gamelkoorn GJ, van de Ende MB, Sminia T: Non-lymphoid cells of bronchus-associated lymphoid tissue of the rat in situ and in suspension. *Cell Tissue Res.* 1985; 239:177-182.

174. van Rooijen N: The "In situ" immune response in lymph nodes: a review. *Anat Rec.* 1987; 218:359-364.

175. VanRooijen N: Antigen processing and presentation *in vivo*: the microenvironment as a crucial factor. *Immunol Today.* 1990; 11:436-439.

176. Waldman RH, Henney CS: Cell-mediated immunity and antibody responses in the respiratory tract after local and systemic immunization. *J Exp Med.* 1971; 134:482-494.

177. Wallace FJ, Cripps AW, Clancy RL, Husband AJ, Witt CS: A role for intestinal T lymphocytes in bronchus mucosal immunity. *Immunol.* 1991; 74:68-73.

178. Weiler H, von Bulow V: Development of optimal conditions for lymphokine production by chicken lymphocytes. *Vet Immunol Immunopathol.* 1987; 14:257-267.

179. Wells CL: Relationship between intestinal microecology and the translocation of intestinal bacteria. *Antonie van Leeuwenhoek.* 1990; 58:87-93.

180. Wells SL, Jechorek RP, Erlandsen SL: Evidence for the translocation of *Enterococcus faecalis* across the mouse intestinal tract. *J Infect Dis*. 1990; 162:82-90.

181. White RG, French VI, Stark JM: A study of the localisation of a protein antigen in the chicken spleen and its relation to the formation of germinal centres. *J Med Microbiol*. 1970; 3:65-83.

182. White RG, Henderson DC, Eslami MB, Nielsen KH: Localization of a protein antigen in the chicken spleen: Effect of various manipulative procedures on the morphogenesis of the germinal centre. *Immunol*. 1975; 28:1-21.

183. Wilkie BN: Respiratory tract immune response to microbial pathogens. *J Am Vet Med Assoc*. 1982; 181:1074-1078.

184. Wolf JL, Bye WA: The membranous epithelial (M) cell and the mucosal immune system. *Ann Rev Med*. 1984; 35:95-112.

185. Woodruff JJ, Clarke LM, Chin YH: Specific cell-adhesion mechanisms determining migration pathways of recirculating lymphocytes. *Ann Rev Immunol*. 1987; 5:201-222.

186. Yagihaski T, Tajima M: Antibody responses in sera and respiratory secretions from chickens infected with *Mycoplasma gallisepticum*. *Avian Dis*. 1986; 30:543-550.

187. Yamamoto R: Infectious coryza. in: BW Calnek, (ed) *Diseases of Poultry*, Iowa: Iowa State University Press, Ames. 1991; pp 186-195.

188. Yoder HW, Jr: Mycoplasmosis. in: BW Calnek, (ed) *Diseases of Poultry*, Iowa: Iowa State University Press, Ames. 1991; pp 198-235.

ACKNOWLEDGMENTS

This work could not have been accomplished without the help of many, many other people.

First of all I'd like to thank my major professor, Lawrence H. Arp, for his willingness to take a chance on a non-traditional graduate student whose full attention could not always be devoted to her graduate work. He taught me to design research that was meaningful, rigorous, and - more practically - fundable and publishable.

I also wish to thank the other members of my graduate committee for their contributions: Ronald K. Myers for sharing his time in thoughtful discussions and his enthusiasm for interesting results; David F. Cox for making statistics such a humane experience; Marit Nilsen-Hamilton for re-igniting my interest in basic cell biology; and James A. Roth for his support and encouragement.

I am one of the few women in science fortunate enough to have had a female mentor. Special thanks to my friend and mentor, Susan Carpenter, for keeping me focussed and for helping me to see the big picture. Thanks, too, for making me a part of the college research family by encouraging my participation at Work in Progress.

Technical aspects of the studies in this dissertation were demanding and time-consuming; they could not have been carried out by myself alone. I wish

to thank my late friend Kay Pierce for teaching me the fine points of excellence in histological techniques. Thanks to Jill Stevens and Becky Smith for providing me with literally thousands of tissue sections for immunocytochemical staining. Special thanks go to Elise Huffman for making lab work fun. Elise's outstanding technical skills were of immeasurable value in the uptake experiments and immunocytochemistry.

I also wish to express my appreciation to veterinary students Sarah Allmon and Traci Koeppel. Sarah cheerfully carried out two of the most tedious parts of the immunocytochemical studies; she stained most of the sections and then had to count positive cells in them. Traci learned to cut beautiful orientation sections for transmission electron microscopy and provided general technical assistance as needed.

Very special thanks go to Dawne Buhrow for the time and energy she unselfishly gave in preparing this dissertation. And thanks, Dawne, for being happy to see me each time I came to your office door with the "last" set of corrections.

I could not have completed my Ph.D. program without the understanding and support of faculty members in the Department of Veterinary Pathology. Although I tried to keep my graduate work from interfering with my job, this was not always possible. I am thankful for the privilege of being allowed to finish my education while continuing to work.

And finally, I'd like to thank my friends for their emotional support (and chocolate), especially during the last year. Thanks, Monica, for Do-Biz cookies to get me through the long days. Thanks, Susan, for basketball tickets, Sunday brunches, and comfort food at the Country Kitchen.