Studies on immune responses to Bordetella avium in turkeys

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Studies on immune responses to *Bordetella avium* in turkeys

Suresh, Poosala, Ph.D.

Iowa State University, 1994
Studies on immune responses to *Bordetella avium* in turkeys

by

Poosala Suresh

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY

Department: Veterinary Pathology
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For the Graduate College

Iowa State University
Ames, Iowa

1994
to

Anu, Vishy, Arvind, Anil, Nithin, Deepika,

my uncle

and

my beloved mom and dad
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ABSTRACT

Systemic and mucosal humoral immune responses to an experimental \textit{B. avium} infection were evaluated by using an ELISA technique. The three isotypes of immunoglobulin, IgG, IgA and IgM, were detected in serum, tracheal washings and lacrimal secretions in response to an intra-nasal and intra-ocular bacterial challenge of 1-day-old turkeys. The IgM response was the earliest, starting at 2 weeks and reaching a peak at 4-5 weeks after infection. The IgA response was early, steady and lower in comparison. The IgG response was prominent and long lasting. The IgG response started at 3 weeks, reached a peak at 5-6 weeks and slowly declined by 8 weeks. The total immune response declined by 8 weeks as the clearance of \textit{B. avium} approached completion. In this study, the cumulative antibody titers closely corresponded with decreasing numbers of bacteria.

The transfer of parenterally administered IgG was assayed in turkeys using \textit{B. avium} specific IgG and ELISA as a model. Purified IgG, specific to \textit{B. avium} was injected and samples of serum and tracheal washings were collected and assayed by ELISA at various time intervals. The IgG intravenously injected was detected in tracheal washings as early as 5 minutes PI, with peak levels occurring 10 minutes post-injection (PI). A rapid decline in serum IgG from 10 to 60 minutes PI was followed by a more gradual decline to baseline levels by 24 hours PI. The levels of IgG in serum, tracheal washings and lacrimal secretions were closely corresponding after 10 minutes PI. These results indicate rapid transfer of IgG from the blood to
mucosal secretions.

The role of parenterally administered IgG in the colonization and clearance of *B. avium* from the tracheal surface was assayed. The IgG prevented colonization and also aided in the clearance of *B. avium* from the tracheal surface. This study showed that IgG, transferred from the bloodstream to mucosal surfaces, could inhibit colonization and promote clearance of infection.

A monoclonal antibody based-latex bead agglutination test was developed to differentiate *B. avium* from *B. bronchiseptica* and *B. avium-like* bacteria. All 40 isolates of *B. avium* tested showed a positive reaction with this test. None of the 24 *B. avium*-like isolates showed a positive reaction. Out of 17 *B. bronchiseptica* isolates, two had minor cross reactions.
GENERAL INTRODUCTION

The disease bordetellosis in turkeys is a problem for turkey producers throughout the United States and other countries (35,38,90). Although the disease itself is not responsible for mortality in a flock, secondary infections may lead to death. Due to the dearth of knowledge, in general, about humoral immunity in turkeys exposed to *Bordetella avium*, there is no effective vaccine available. Even the vaccines that are or have been used are not effective due to the age of the bird at administration or the antigenic composition (92).

Many studies on immune responses to various respiratory pathogens in mammals and birds have shown that antibodies are effective in either preventing infection or promoting recovery from disease (13,55,62,139,197). There have been few studies that clearly determined the role of each isotype of immunoglobulin in a respiratory mucosal disease. Several studies have been done to detect various antibodies in birds (13,55,62), but none of these gave a detailed picture of the role of each isotype. In birds, these kinds of studies have been sparse and information related to protective effects of antibody isotypes against a respiratory pathogen are even more sparse.

It has been known for at least three decades that birds are capable of producing all three isotypes - IgA, IgM and IgG - in response to a disease on the mucosal surfaces (116). Recent studies have shown the presence and importance of
mucosa-associated lymphoid tissue (MALT) at various locations (28,29, 86). Even though, the role of these lymphoid tissues in the respiratory tract has not been fully explored, present knowledge suggests that they might be very important for the immune response. In response to an antigenic challenge, these lymphoid tissues undergo marked hypertrophy, with increased cell numbers and probably germinal centers, conveying a morphologic basis for the humoral immune response (68). Similarly, previous studies have shown that the Harderian gland has increased numbers of plasma cells and that it plays an important role in immunity against diseases of the upper respiratory tract (176).

Current knowledge about the source of each antibody isotype in mammals may also apply to birds. The main sources of IgM and IgA seem to be local cell populations in the lamina propria of mucous membranes of certain tissues. The main source of IgG found at the mucosal surface seems to be serum (161). However, none of these sources are mutually exclusive, and there is no direct evidence for a single antibody being responsible for the immunity. A study in chickens demonstrated the transfer of IgG to lacrimal secretions (182). The relatively smaller size of IgG makes it capable of undergoing simple diffusion (114). However, IgM and IgA have secretory components which facilitate their transport across epithelial cells. Even though the role of IgA in mucosal infections is considered important, the role of IgM and IgG cannot be ignored as there is no direct evidence linking absence of IgA with susceptibility to disease. The overall picture with the currently available knowledge is that systemic and mucosal humoral immune responses together play an important
role in protecting an animal from respiratory infections.

An understanding of the immune response to *Bordetella avium* in turkeys is an important step in understanding the role of each immunoglobulin isotype during infection. Also, a knowledge of the source of each immunoglobulin should shed light on mucosal immunology. A knowledge of the role of immunoglobulin isotypes in turkey bordetellosis may lead to better vaccination strategies and serologic techniques.

Apart from understanding the immune aspects of this disease, there is also an urgent need for devising a simple diagnostic test to detect *Bordetella avium* and differentiate it from closely related bacteria like *Bordetella bronchiseptica* and *B. avium*-like organisms. This kind of test could make the laboratory diagnosis of bordetellosis more rapid and efficient.

**Objectives of Dissertation Research**

The detection of a humoral immune response to *Bordetella avium* in turkeys suggests that antibodies may be important in the defense against this disease. Hence, the objectives of this research were to:

1) Determine the onset, duration, and quantities of immunoglobulin isotypes produced during infection, 2) characterize the temporal aspects of IgG transport from serum to mucosal surfaces, 3) analyze the importance of IgG in preventing colonization or clearing of *B. avium* from the tracheal surface, and 4) devise a simple diagnostic test for identification of *B. avium*.
Dissertation Organization

This dissertation consists of three manuscripts; each one is presented in the format of the journal Avian Diseases. Preceeding the papers is a literature review; following the papers is a general summary and discussion. References cited in the general introduction and literature review follow the general summary and discussion. The first manuscript to be published in Avian Diseases is in press. The appendix is a complete research article published in Avian Diseases. Dr. Suresh was the principal investigator and first author for each phase of research and corresponding manuscript.
Anatomy of the Avian Immune System

The structure and organization of lymphoid tissue in birds differs significantly from that in mammals. Major differences are the absence of lymph nodes and the presence of a special lymphoid organ, the bursa of Fabricius in birds. The bursa is important in the development and differentiation of B-cells (163). In spite of the differences in structural organization of lymphoid tissues between mammals and birds, the functional aspects of lymphoid cells and peripheral lymphoid organs are similar (153). Small lymphocyte accumulations (0.5 mm in diameter) are present along lymphatic vessels in birds (153). Though these small nodules enlarge and develop germinal centers when stimulated by antigen, they lack the size and complexity to function as lymph nodes in mammals (130). However, the enormous number of these nodules in parenchymal tissues such as bone marrow, skin, liver, lung, kidney, pancreas, endocrine glands, larynx and trachea compensate for the lack of distinct lymph nodes (153).

Primary Lymphoid Organs

Thymus

The mature thymus is a 12- to 14- lobed structure located close to the jugular veins (163). The lobules of each lobe are further separated into cortical and
medullary zones. A dense epithelial cell network and Hassal's corpuscles are found in the medulla. Also present in the avian thymus are lymphoid cells of various sizes, epithelial cells, macrophages and myeloid cells (78). Evaluation with flow cytometric analysis revealed that thymic cells are composed of large blast-type cells, lymphocyte sized thymocytes and small thymocytes. In contrast to the mammalian thymus, the avian thymus has afferent lymphatics and has the potential to act as a peripheral lymphoid organ permitting B-cell traffic after hatching (121). Mature plasma cells are found after 4 weeks (4, 96). In response to antigenic stimulation, specific antibody presenting cells appear in the avian thymus (103). Chicken thymic extracts induce the specific T-cell allo-antigen Th-1 on embryonic bone marrow cells, suggesting the presence of thymic factors similar to those in the mammalian thymus (39).

The Bursa of Fabricius

This follicular epithelial lymphoid organ is located dorsal to the distal end of the cloaca (163). The horizontal infoldings and plicae formed by the developing epithelial cells are colonized by lymphoid cells which define cortical and medullary regions. Each bursal follicle is populated by approximately 10,000 lymphoid cells. The bursal medulla contains small lymphocytes, plasma cells and macrophages (140). The bursa of Fabricius, in addition to its role as a central immunological organ, also functions as a peripheral lymphoid organ. Antigenic material given by various routes can induce an antibody response from antigen presenting cells (APC) in the bursa (190). The lymphoepithelium covering the bursal follicles is specialized for antigen
uptake (37). This lymphoepithelium is capable of uptaking and transporting tracer particles by pinocytosis. After localizing in bursal follicles, these tracer particles are further distributed to other regions of the bursa by phagocytic cells (21). Also observed in the bursa are T-cells (128) in special T-dependent areas with an APC population (142). A dense lymph vessel network with lymphoid cells is present outside the follicles of the bursa (65). The lymph flow is away from the bursa, and it has been suggested that efferent lymph vessels are involved in the transport of cells from the bursa to peripheral tissues (65).

Secondary Lymphoid Organs

Spleen

The structure of the avian spleen is similar to its mammalian counterpart except for the ellipsoidal reticular cells arranged in sheaths around penicillary arteries (144). Lymphoid cells colonize the spleen at 10 to 11 days of embryonic life in birds (5). The red pulp and white pulp start to develop at 8 and 12 days of incubation, respectively (153,166). Periarterial tissue surrounding the central arteries and arterioles is dominated by T lymphocytes. Germinal centers are located within the periarterial lymphoid tissue located close to the central artery at a site where it branches into penicillary arterioles (179). B-cells are present at day 12 of incubation and IgG and IgA positive cells are present at the time of hatching (153). The lymphoid structure undergoes major development after hatching in response to antigenic stimulation. The spleen produces antibodies mainly against blood-borne
antigens (189). There is direct correlation between the generation of splenic germinal centers and bursal development, both reaching peak maturity at 4 to 5 weeks of age. The formation and presence of germinal centers requires cooperation between B- and T- cells (179). B cells in the germinal centers are considered to represent a pool rather than B cells differentiating into plasma cells (193).

**Mucosal Lymphoid Tissues**

Mucosa-associated lymphoid tissue (MALT) is present along mucous membranes throughout the body, particularly the digestive and respiratory systems. The Harderian gland, conjunctiva-associated lymphoid tissue (CALT), lymphoid nodules associated with the nasolacrimal gland, bronchus-associated lymphoid tissue (BALT), Peyer's patches, the cecal tonsil, and Meckel's diverticulum are the components of avian MALT (17,73). In birds, MALT is highly developed and may be functionally similar to mammalian lymph nodes in responding to antigen. Avian MALT is strategically located at sites where there is high exposure to environmental antigens. Some component tissues of MALT, such as Harderian gland and Meckel's diverticulum contain large numbers of plasma cells primarily involved in local antibody production (138). Some other components of avian MALT, such as CALT have more lymphoid nodules and fewer plasma cells, presumably these tissues are involved in the initiation of immune responses, rather than local antibody production (22). This kind of functional diversity in the immune responses of MALT makes it an important entity for the immunologist and even more important for the bird itself.
Gut Associated Lymphoid Tissue (GALT)

The presence of GALT has been demonstrated throughout the gastrointestinal tract in mammals and birds. GALT refers to various lymphoid tissues in the oral cavity, pharynx, small intestine, cecum, bursa and rectum. Hence, GALT contributes substantially to the total amount of lymphoid tissue in the body. The morphologic changes associated with changes in cell populations in GALT in response to antigens suggests their role in immune responses. The lymphoid nodules in the lamina propria of these organs contain germinal centers which are considered thymus dependent. The phagocytic cells and lymphoid cells considered to be bursa-dependent are present in the epithelial and sub-epithelial zones (153).

Avian Peyer’s patches are structurally similar to those in mammals (22). The Peyer’s patches, located in the distal ileum, are characterized structurally by flattened intestinal epithelium, lack of goblet cells, and thickened villi. Both germinal centers and diffuse lymphoid tissue are present in Peyer’s patches (43). Most B cells in Peyer’s patches have surface IgG with some IgM or IgA (43). The number of Peyer’s patches increases up to 16 weeks of age and then decreases to a single residual patch (22).

Meckel’s diverticulum is the embryologic yolk sac remnant that separates the jejunum from the ileum (146). Infiltration of lymphoid cells begins at the age of 2 weeks and attains maturity, as indicated by functional germinal centers, at 5 to 7 weeks of age (179). Large numbers of plasma cells are present in the Meckel’s diverticulum; like the Harderian gland it remains functional until 20 weeks of age.
The germinal centers in the cecal tonsils can either be completely encapsulated or incompletely encapsulated (143). The appearance and maturity of the cecal tonsils is dependent on the antigenic stimulation in the intestine (141). Bursectomy reduces the number of germinal centers in the cecal tonsils (102).

**Bronchus Associated Lymphoid Tissue (BALT)**

BALT is an important mucosal lymphoid tissue. It has been identified in several species including rabbits, rats, mice, guinea pigs, pigs, chickens, turkeys, dogs and humans (30,68). It is present at the bifurcation of airways where deposition of antigenic material occurs. BALT was first characterized 20 years ago (30,31). These authors mentioned that, chickens had more lymphoid tissue in the trachea compared to other species mentioned above. Also, in contrast to mammalian BALT, avian BALT occurs as prominent subepithelial lymphoid nodules, raising the surface epithelium and protruding into pulmonary bronchial lumina (30,68,187). A complete description of BALT in turkeys has been published (68,187). BALT of turkeys is present along the primary intrapulmonary bronchus. It is specifically associated with longitudinal mucosal folds and the openings of secondary bronchi (68). Light and electron microscopic studies of BALT in normal turkeys at various ages showed that it resembled other MALT. The lymphoepithelium of BALT in chickens varies from primarily squamous in 1 week old chicks to ciliated columnar cells in 6 week old chicks (68). In studies involving turkeys infected with *Bordetella avium*, BALT
nodules were more numerous and more widely distributed than in normal birds (72,187). BALT nodules in all ages of turkeys have a dome-shaped ciliated epithelial surface interrupted at the dome apex by patches of non-ciliated cells (68). Ultrastructurally, the same study showed discontinuities in the basal lamina associated with infiltrating lymphocytes. This shows that turkey BALT may be specialized for antigen uptake and the associated immune response (68).

**Paranasal and Paraocular Lymphoid Tissue**

Birds have a closed, bony orbit, sinuses and paraocular glands. There are several accumulations of lymphoid tissue in the paraocular and paranasal areas (180). Of the paraocular glands, the Harderian gland contains a dense infiltration of plasma cells. Some workers observed that these infiltrates along the ducts were primarily small lymphocyte nodules with germinal centers (19). The Harderian gland is a retrobulbar, tubulo-alveolar gland located in the orbit medial to the eyeball (163). Its secretions empty into the conjunctival space behind the nictitating membrane by a single duct. The Harderian gland is thought to contribute to the immune function of the orbital, nasal, and upper respiratory tract tissues.

The Harderian gland is divided into lobules composed of columnar epithelium supported by fibrovascular interstitial septa. It is surrounded by a thin connective tissue capsule (194). Ultrastructurally, four types of epithelial cells are recognized (164). The interstitium of the Harderian gland is infiltrated by lymphoid cells between 17 to 18 days of embryonic development. Lymphocytes, macrophages,
myoepithelial cells and heterophils are also present in the gland (19,167).
Plasmablasts forming desmosome-like junctional complexes with each other and macrophages have been observed ultrastructurally (167). Plasma cell infiltration begins at the time of hatching (194) and increases with age (176). Studies in chickens have shown early infiltration of IgM-positive cells at 2 to 4 weeks (6). IgG- and IgA-positive cells increased from 4 to 9 weeks in similar studies (6,176). Mansikka et al., (1989), detected all three isotypes of immunoglobulins in the Harderian gland plasma cells even in the absence of local stimulation, as determined by Northern hybridization, in situ hybridization and ELISA (123).

Numerous studies have shown antibody producing capability by the plasma cells in the Harderian gland. Conjunctival inoculation with Newcastle disease virus, infectious bronchitis virus (IBV) and Mycoplasma gallisepticum induced production of specific homologous antibody in the Harderian gland (158) in contrast to no detectable antibody responses after parenteral inoculation. One study showed lymphoid follicle formation at 14 to 21 days in the Harderian gland after parenteral inoculation with IBV (57).

**Conjunctiva Associated Lymphoid Tissue (CALT)**

CALT is morphologically similar to GALT and BALT (17,47,73) and contains multiple nodules of lymphocytes beneath the palpebral conjunctiva of the lower eyelid (17,47). CALT might function as a site of antigen uptake and initiation of a local immune response (17,47,48,72). Infection of turkeys with B. avium increased
the number of germinal centers in CALT (72). Studies of CALT in rabbits (77),
humans (26,76) and birds (19,73,83,153) confirmed existence of plasma cells
producing secretory IgA. CALT in turkeys seems to resemble morphologically that in
rabbits. CALT in turkeys has been proposed to function as a site of antigen uptake,
processing and presentation (74).

**Lymph Nodes**

Lymph nodes, classically present in mammals, are not present in most avian
species including turkeys. Instead, there are regional concentrations of lymphoid
tissue throughout the organs, tissues and lymphatic vessels (63). These lymphoid
tissues are primarily made up of small lymphocytes. Lymphoid accumulations in
chickens probably function like mammalian lymph nodes and have been observed
along the posterior tibial, poplitial and lower femoral veins (145). These femoral
lymph nodules have afferent and efferent lymphatics, peripheral germinal centers and
internal sinuses equipped with valves to direct the flow of the lymph.

**Mucosal and Systemic Humoral Immune System of Poultry**

**Avian Immunoglobulins**

The major difference between avian and mammalian immunoglobulins is the
presence of a major serum 7S immunoglobulin heavy chain that is about 10 Kd
heavier than the mammalian gamma-chains. Immunoglobulin isotype diversity in
birds, is structurally limited to only three isotypes (7S IgG, IgM and IgA) with no subclasses (25).

**Immunoglobulin G (IgG)**

The 7S IgG of chickens has several characteristics that resemble human IgG; like susceptibility to proteolytic digestion, aggregation, and an H chain larger by one domain. This IgG has a total carbohydrate content of 6.0% (1,95), weighs 165 to 180 Kd (118) and has a sedimentation coefficient of 7.3 to 7.4 S (26). X-ray small-angle scattering studies have shown that the chicken IgG molecule is less flexible in comparison to mammalian IgG. The concentration of chicken IgG in normal serum ranges from 5-7 mg/ml (116). The metabolic half-life of chicken IgG is 3 days in neonatal (152) and 1.5-4.3 days in adult birds (119). In contrast to mammalian species, IgG in the chicken is the predominant immunoglobulin in the tracheal secretions (48).

**Immunoglobulin M (IgM)**

Chicken IgM is similar to its mammalian counterpart in many respects. Chicken IgM is found in serum as a pentamer and has a molecular weight of about 890 Kd and a sedimentation coefficient of 17 S (24,118). Monomeric IgM has also been reported in fresh serum treated with $10^{-3}$M iodoacetamide (116). The carbohydrate content is 6.6% (11). The metabolic half-life of chicken IgM is 1.7 days (118). Mockett observed common IgM determinants in different avian species (134).
A secretory component associated with IgM has been detected as a free component in egg white (157). Mockett (1986), detected IgM in bile and speculated that it might also be transferred from blood to bile by binding to secretory component (133). Peppard et al. have shown the existence of a functional homologue of mammalian secretory component in chickens and biochemically characterized it (154,155). The concentration of IgM in normal chicken serum is 1-2 mg/ml (116).

**Immunoglobulin A (IgA)**

Birds have abundant polymeric IgA in bile and intestinal secretions (147, 116). There is a relatively higher concentration of IgA in secretions on all mucosal surfaces, in comparison to the serum. In chickens, IgA forms less than 4% of total serum immunoglobulins (34). Polymeric IgA is present in bile and intestinal secretions, saliva, tears and tracheal washings of turkeys (60). Structurally, avian IgA resembles mammalian IgA, although this has not been rigourously tested by molecular methods (115). It occurs in monomeric and polymeric forms, with the latter dominating in secretions and serum (34,120). Only 20% of serum IgA is monomeric, the other 80% exists in dimeric or multimeric forms (116). The molecular weight and sedimentation coefficients range from 350-900 Kd and 9-16 S, respectively (151,157). Avian IgA has a J- chain (136) and a secretory component (25). Genome sequencing may be helpful in clarifying the degree of homology of avian IgA to other mammalian IgA. The concentration of IgA in serum, intestine and bile is 0.3 to 0.645, 1.6 and 3.15 mg/ml, respectively (116).
Mammalian IgA has been functionally credited with inactivating bacterial enzymes and toxins, antibody-dependent cell mediated cytotoxicity (ADCC), preventing bacterial attachment to host cells and virus neutralization. Specific details about the function of IgA in birds are lacking. Studies on the immune function of avian IgA in experimental infections with Newcastle disease virus and infectious bronchitis virus (IBV) were inconclusive (150). IgA was expected to be related to viral neutralizing activity in tracheobronchial secretions after vaccination with modified-live IBV, but a challenge infection provoked an IgG response in secretions with a minimal IgA response. Other immunoglobulin isotypes may substitute for the protective functions of IgA at mucosal surfaces in IgA deficient humans and birds as IgM can also associate with secretory component (51,114).

Antibody Response in Avian MALT

Even though MALT occurs at various anatomic sites, the function is rather uniform. This function includes antigen uptake, processing, and antibody production at the mucosal surface. A number of papers have reviewed MALT functions (28,29,33,86).

Antigen Uptake and Presentation

The first and most important step for generating an immune response is antigen uptake. This uptake mechanism has been evaluated primarily using carbon particles, latex beads or tracer macromolecules like ferritin, mycobacteria and horse
radish peroxidase (HRPO). The lymphoepithelium on the surface takes in the antigen by a process of pinocytosis (37). Carbon particles have been demonstrated histologically crossing the lymphoepithelium of rabbit appendix and mouse Peyer’s patches followed by localization in the mesenteric lymph nodes (85,107). Latex beads have also been used in similar studies in rats, mice and dogs (117,192). Apical vesicles are present in lymphoepithelial cells of mammalian BALT, especially when an active immune response has been stimulated (160,188). The uptake of HRPO by M-cells has been elucidated ultrastructurally in mouse Peyer’s patches (148,164). Studies have shown that tracer particles are taken up by BALT lymphoepithelium as well (188). Studies using ferritin and \textit{B. avium} showed that both ciliated and non-ciliated cells of BALT are capable of uptake (70,72,75). These studies showed ultrastructurally that these particles appear in organelles associated with endocytosis. Also, uptake of tracer particles like carbon, iron oxide and latex beads by lymphoepithelial cells in CALT was shown by a recent study (74).

Some studies have shown that both bronchial and nasal epithelial cells were able to present antigen to T cells and stimulate mixed lymphocyte reactions (MLR) in allogeneic systems (109). Also, studies on GALT have concluded that absorptive enterocytes constitutively express MHC-II (125) and are capable of presenting antigen and stimulating T cell proliferation, and stimulating IL-2 production (36,108,125).
Immune Response in MALT

Various immunocytochemical studies have shown that B lymphocytes and T lymphocytes of both T-helper and T-cytotoxic/suppressor subsets are present in BALT nodules, often in localized compartments (40,170). Distinct T- and B- lymphocyte regions have been demonstrated in the BALT of poultry (69,106). Antigen presenting accessory cells are of different kinds in the lymphoid regions of BALT and in the germinal centers of chicken BALT (106). Macrophages are distributed throughout the lung parenchyma and BALT in chickens (46,106). Several studies have demonstrated the appearance of germinal centers in the nasolacrimal duct, GALT, tonsils and BALT of chickens between 2 and 3 weeks of age (10,19,105,177). Germinal centers are prominent in the avian BALT, supporting the idea that this tissue may functionally compensate for the lack of lymph nodes in the bronchial region in birds. The presence of germinal centers might simplify the model for antigen initiated antibody response. This model indicates that virgin or naive B cells first encounter the antigen in T cell zones composed of T-helper lymphocytes, B lymphocytes and interdigitating dendritic cells (IDC). These antigen primed B cells migrate to primary follicles, where they enter the phase of exponential cell division. Affinity maturation also takes place at this step and only B cells with high affinity are allowed to proliferate. Among these B cells, some proceed to small memory B cells while others differentiate into plasma cells producing immunoglobulins. Thus, the development of germinal centers seems to be an evolutionarily conserved plan as evidenced by its presence in several mammalian and avian species, like the model for cellular
interactions between T and B lymphocytes and APCs (186). The development of
BALT depends on the new antigens encountered everyday and on the genetic
variability among animals (8).

Secretory IgA has been considered the primary humoral defense antibody on
the mucosal surfaces. The dimeric or polymeric configuration associated with the
secretory chain facilitates the mucosal transport of IgA (185). Studies in rabbits and
humans have shown that B- immunoblasts in the mucosal lymphoid organs are
mostly committed to the production of IgA (52,98). An effective presentation of
processed antigen fragments to immature B cells turns on antibody production (113).
Isotype switching due to different interleukins might be one mechanism of secreting
specific antibodies. The committed B cells leave the lymphoid tissue and migrate to
various mucosal surfaces as immunoglobulin-producing plasma cells. The presence of
processed antigen, APCs, interleukins, receptors and other vascular features contribute
to the localization and distribution of antibody secreting cells at various mucosal
surfaces (27,54). Also, it is known that the presence of secretory component in IgA
permits transport of this antibody through the cytoplasm of the the epithelial cells
(162,181). Several studies have identified IgG and IgM in the secretions from
mucosal surfaces.

Initiation of antibody responses at various sites other than the site of antigen
administration is an effective component of mucosal and systemic immune responses.
This is made possible by the selective localization of T and B lymphocytes around
the body (27,97,196). This selective localization is termed homing and has been
reviewed extensively. The high endothelial venules (HEVs) in lymph nodes act as binding sites for the entry of lymphocytes expressing specific surface adhesion molecules (196). This process of homing dictates the distribution of lymphocytes to different tissues (50). The tissue origin of lymphocytes influences their subsequent distribution to distant sites, thus lymphocytes from BALT tend to home to the lungs and respiratory mucosa, lymphocytes from GALT tend to home to the gastrointestinal tract (45,131). These homing patterns and subsequent interactions dictate the antibody response at different mucosal sites. These studies support the presence of a distinct humoral immune mechanism of MALT in response to antigens.

Respiratory Immunity in Poultry

Potential role of MALT in Respiratory Immunity in Birds

Because avian MALT develops at strategic sites of exposure to external antigens, it is likely that MALT has an important role in the immune response. In the respiratory tract, the BALT is located at the airway bifurcation points where there is optimal deposition of antigens due to changes in airflow direction and velocity (32). This suggests that the lymphoepithelium of BALT would be one of the initial contact points for airborne pathogens. The two main functions of BALT epithelium are uptake and transport of antigens. Additionally, lymphoepithelial cells may have a role in antigen presentation. Antigen transported across the lymphoepithelium may interact with the underlying lymphoid cell population leading to a mucosal immune response.
Knowledge of avian BALT, suggests that both B- and T- lymphocyte compartments are present in BALT nodules and both T- helper and T-cytotoxic / suppressor cells are involved (10,106). Also, accessory cells, including follicular dendritic cells (FDC) and macrophages, are present in avian BALT (46,106,188). Unlike mammalian BALT, avian BALT has prominent germinal centers (31), in support for a humoral immune response function (122). Though there is little experimental documentation about high endothelial venules (HEV) and lymphocyte homing in BALT of chickens, they do occur in CALT and GALT (22,73). However, HEV are located at the periphery of BALT nodules, mostly at the T and B lymphocyte region interface. Hence, the evidence points to a potential role of avian MALT in the humoral immune response and lymphocyte mediated immune help such as cytokine production, immune response enhancement, or immunoglobulin switch.

**Humoral Immunity**

Antibody has a major role in protecting the host against infection with various pathogens. It can act in different ways; either by preventing attachment and colonization of pathogens to host cells or by neutralizing toxins and other harmful molecules. Indirectly, antibody aids in the clearance of bacteria by phagocytes and antibody-dependent cell mediated cytotoxicity (ADCC). Several papers have suggested a role for immunoglobulins in protection against respiratory pathogens of poultry, such as in infectious bronchitis virus (IBV), Newcastle disease virus (NDV), influenza virus, *Mycoplasma gallinarum*, *Escherichia coli* and *Bordetella avium* (13,55,
The antibody in the respiratory tract, primarily detected in tracheal secretions, may be secreted by the local plasma cell population or be transported to the respiratory mucosal surface via the bloodstream (110). IgG diffuses most readily from serum to the airway mucosa due to its small size (161). In contrast, IgA and IgM found on respiratory mucosal surfaces are chiefly derived from the local plasma cell population rather than from serum. Transport of serum IgA and IgM to the respiratory mucosal surface has not been reported in avian species. Apart from these origins, antibody may also be found in mucosal secretions due to capillary permeability changes associated with local inflammation.

The potential role of each immunoglobulin isotype has not been discerned for the majority of mucosal diseases in mammals as well as in birds (49). However, IgA is considered to be the most important immunoglobulin isotype at mucosal surfaces in mammals. Since the absence of IgA does not result in profound susceptibility, other immunoglobulin isotypes must be important as well (114). The data for birds regarding the role of each immunoglobulin isotype is very limited.

**Cell Mediated Immunity**

Cytotoxic lymphocytes, macrophages, natural killer (NK) cells and granulocytes are the components of cell mediated immunity (CMI). In contrast to mammalian NK cells, in birds NK cell activity is not restricted to a single cell population.
In contrast to mammals, the lung lavage from birds is predominated by heterophils with fewer lymphocytes and macrophages (80). Avian heterophils lack myeloperoxidase and catalase enzymes and thus are incapable of killing several kinds of bacteria, hence the birds may be more susceptible to respiratory disease (183). However, lysozyme and cationic enzymes in heterophil granules are capable of killing bacteria, and avian heterophils are capable of protecting birds against several respiratory pathogens (183,184). In addition, the epithelial cells lining various gas exchange regions are capable of phagocytosing foreign material.

The potential role of avian NK cells in respiratory defense has not been explored fully. However, some reports have indicated that NK cells may mediate cytotoxic activity against infectious bronchitis virus and Marek’s disease (159). NK cells in birds act in a similar manner to mammalian counterparts; that is, they carry out their cytotoxic activity independently of major histocompatibility restriction (71).

In addition to non-specific cell mediated cytotoxicity, certain cells are capable of specifically killing cells (target cell) expressing surface antigens (tumor or viral). Cytotoxic T lymphocytes (CTLs) kill cells expressing foreign antigens complexed with MHC antigens of Class I. Also, cells such as NK cells, macrophages and granulocytes express Fc receptors and thus recognize antibody complexed with foreign agents (ADCC). Avian macrophages and heterophils (61,178) express Fc receptors, but ADCC has not been explored for non-respiratory pathogens (159,169). Lymphocyte blastogenesis and lymphocyte migration inhibition (LMI) tests indicate a potential role of CMI in protecting chickens against infectious bronchitis virus (IBV) (55).
Antibody Transport

In mammals, the three main immunoglobulin isotypes, IgG, IgA and IgM, are capable of diffusing from serum into airways in small amounts (110). However, IgG, in view of its relatively smaller size (161), is more abundant in airways than IgM and IgA (126).

IgG transport in human and canine respiratory epithelium, has been shown to occur by a receptor-mediated mechanism (132). This mechanism has not been fully explored in avian respiratory epithelium. A major portion of polymeric IgA and very little of IgM is transported by binding to polymeric Ig receptor (pIgR) on epithelial cells (9). This transport mechanism is seen in bronchial epithelial cells of mammals (32). No knowledge about such a transport mechanism is currently available for avian species.

Immunoglobulins have been demonstrated in respiratory secretions of chickens in response to agents such as Newcastle disease virus and avian paramyxovirus (2,62,66). Several studies in birds showed that the amount of IgG in nasal, tracheal and tracheobronchial secretions exceeds that of IgA (23,48,67,82). These studies also speculated that there may be selective mechanisms for IgG transport in birds. Paraocular fluids contain antibodies of different isotypes chiefly secreted from the plasma cells in regional glands (56). Plasma cells in the Harderian gland have been shown to secrete infectious bronchitis virus-specific IgA as demonstrated by immunofluorescence studies (56). Mucosal secretions contain both IgA and IgG, although IgA initially is the chief immunoglobulin (2,66). In virus infected chickens,
IgG overtakes other immunoglobulins and inhibits replication in the trachea for 4 weeks (66). In poultry, IgA is found in lesser quantities than IgG in tracheal secretions, saliva and tears (48,116). IgA:IgG in serum and secretions indicate that most of the IgA in the respiratory tract is locally produced (48). IgA is selectively enriched in tracheal washings in comparison to serum, thus signifying an important role for IgA, however, studies on the role of IgA in neutralizing Newcastle disease virus or infectious bronchitis virus were inconclusive (67,82,150). In the IBV study, a higher dose challenge elicited a prominent IgG response in secretions and little IgA response.

Studies on the distribution of immunoglobulins in the respiratory tract of sheep showed that IgM was the main immunoglobulin seen by immunofluorescence and immunoperoxidase techniques in the nasal and bronchial glands of lambs before suckling (7). The same study showed that in adult sheep, IgA- and IgG- containing cells were in equal numbers in nasal mucosa and IgA cells predominant in the bronchial mucosa and lung. Cytoplasmic staining of bronchial epithelial cells for IgA and IgM was seen most frequently in areas of proliferating epithelium suggesting that immature cells may be engaged in immunoglobulin transport.

Pittard et al. (1977), proposed that the breastmilk macrophage is a potential vehicle for immunoglobulin transport (156). Burton and Smith (1977) showed that endocytosis plays a role in the immunoglobulin transport across the small intestine of new-born pig (44). Devenny and Wagner (1985) proved the ability of capillary endothelial vesicles to transport IgG across the capillary wall. Capillary endothelial
cells were found to endocytose fluorescent labeled IgG via a bulk fluid phase mechanism. Transport of immunoglobulins and other proteins takes place in a bidirectional manner across the peritoneal tissue (58).

Few studies have been conducted on the metabolism and passive transfer of immunoglobulins in turkey hens. A study by Dohms and Saif (1978), showed that IgG is the chief immunoglobulin transferred from hen to the egg. The transport of IgM and IgA into the egg was considered negligible and unimportant (59). Chickens have been passively protected against *Eimeria tenella* infection by parenterally administered monoclonal antibody of IgG\(_i\) isotype (53). Thus, serum antibody has been shown to play an important role in immunity to coccidiosis.

Studies on kinetics of immunoglobulin transport into canine bronchial secretions showed that a small percentage of IgG administered passively is recovered in the secretions indicating transfer of humoral IgG into mucosal secretions (111). Furthermore, the same study showed that IgM administered intravenously was not detected at all in these secretions. I-125 labelled IgG has been shown to be transported across the small intestine of rats (137).

Toro et al. (1993), have shown that peak levels of I-125 labelled IgG administered intravenously into the wing vein of chickens could be detected in the lacrimal secretions within 10 minutes (182). This study also suggested that the IgG level in serum declines rapidly from day one to day 20. Keeping these different interpretations of several studies in view, the role of IgG seems to be important even on mucosal surfaces.
Antibody Catabolism

Studies on the metabolism of immunoglobulins were largely conducted using isotypes labelled with radioactive chemicals. The metabolism of immunoglobulins is similar to other proteins, with increased catabolism occurring during hyperthermia (18). The rate of immunoglobulin synthesis and degradation depends on the class of immunoglobulins. In humans, an increase in the serum concentration of IgG enhances its degradation rate, possibly due to an autoregulatory mechanism. The same principle, however, does not apply to IgM and IgA, and, IgD and IgE are catabolised rapidly even when their concentrations are low. Fab fragments and light chains have very short half-lives while Fc fragments have a half-life equal to the whole IgG molecule. It is useful to remember that more than half of the IgG and IgA molecules are extravascular, while the IgM is largely intravascular (18). The catabolic rate (percentage of intravascular pool broken down per day) of human immunoglobulins were 6.7, 25, 18, 37 and 89 for IgG, IgA, IgM, IgD and IgE, respectively (18). Similar data are not available for avian species.

There are different ways by which antibody concentrations in the body are influenced. Antigen-antibody complexing, internalization and shedding of antibody leads to false interpretation of antibody catabolism studies. This happens in many viral and bacterial infectious diseases and tumors like Burkitt's lymphoma and human colon cancer (168,191). Also, the labelling method used in catabolism studies might complicate the results. In one study by Matzku et al., use of 67-Ga-phenolic aminocarboxylic acid chelate labelled monoclonal antibody demonstrated a major
difference from $^{125}$I-labelled monoclonal antibodies, probably due to longer retention of this conjugate in tissues physiologically involved in antibody catabolism (124). Hereditary errors may also lead to faulty catabolism of immunoglobulins; in myotonic dystrophy an accelerated breakdown of IgG occurs (195).

Liver is the primary organ involved in antibody catabolism (79). However, a study expressing antibody on a concentration basis, showed that spleen accounted for 247% of the saturable compartment/kg; whereas, liver accounted for 25%/kg along with a minimal saturable uptake by bone marrow (64). This same study speculated that saturable uptake may relate to sinusoidal blood supply characteristic of liver, spleen and bone marrow.

A study on the kinetics of the decline phase of the primary response to human serum albumin in the chicken, showed that the antibody levels in the decline period (7-17 days) manifested exponential decay and a constant $t_{1/2}$ of 2.2 days, which according to the authors, approximated that observed with passively administered $^{31}$I-labelled 7S chicken immunoglobulin (88). These data suggest that antibody catabolism in general occurs in all animals and is similar to the catabolism of other plasma proteins. Interpretation of antibody kinetic studies is complicated by various factors influencing the distribution and catabolism of immunoglobulins.
Bordetellosis in Turkeys

Bordetellosis is a highly contagious upper respiratory tract disease of turkeys characterized by sneezing, oculonasal mucus discharge, submandibular edema, collapse of trachea and a predisposition to other infectious diseases (16). The economic loss due to bordetellosis has not been assessed; but, losses due to impaired growth and mortality due to colisepticemia and other secondary bacterial infections might cause losses up to several million dollars annually to the turkey industry in the USA. The disease has also been referred to as turkey coryza, alcaligenes rhinotracheitis (ART), B. avium rhinotracheitis (BART) or turkey rhinotracheitis.

Etiology

The disease bordetellosis is caused by a gram negative bacterium called *Bordetella avium*. The disease has been successfully transmitted to susceptible pouls (172,173). *Bordetella avium* is a motile, strictly aerobic, non-fermenting bacterium (112,174). Morphological, nutritional, physiological, serological, electrophoretic and DNA-RNA hybridization studies confirmed this bacterium as belonging to the *Bordetella* genus. Infection with *B. avium* accompanied by stress is the primary cause of this disease.
Growth Characteristics and Colony Morphology

*Bordetella avium* produces small, compact, translucent, pearl-like colonies on MacConkey's agar. Colonies reach a size of 1 to 2 mm after 24 to 48 hours of incubation at 25°C (174).

Pathology, Clinical Signs and Lesions

*Bordetella avium* tends to be less pathogenic in chickens than in turkeys (135). Natural infection in turkeys is typically recognized in 2 to 6 week old poults (38,90,149). Bordetellosis is transmitted to susceptible turkey poults through close contact with infected birds or contaminated feed or water (172). The incubation period in natural infection ranges from 7-10 days (172); whereas, experimental intranasal and intraocular inoculation results in clinical signs within 4-6 days (11,165).

The bacteria initially adhere to the ciliated epithelial cells of the upper respiratory tract. One review suggested that the pili of *B. avium* are involved in the *in vitro* attachment of the bacteria to the mucosal surface of turkey tracheal explants (101). Colonization and expansion of the bacterial population leads to acute inflammation and mucus discharge from goblet cells causing sneezing and obstruction of the upper airways (84). The progressive sloughing of epithelial cells colonized by *B. avium* leads to loss of cilia and prevents subsequent mucus clearance (11). This damage to the ciliated epithelium is probably due to a tracheal cytotoxin (81). Obstruction of the nasolacrimal ducts causes accumulation of foamy exudate in
the nares and medial canthus of the eye.

The clinical signs of bordetellosis seem to result from local and systemic products of the inflammatory response, soluble bacterial toxins and physical obstruction of the upper respiratory tract (16). The most prominent sign of bordetellosis in a majority of 2 to 6 week old turkeys is sudden onset of sneezing. The profuse mucus discharge from the eyes and nares gives the feathers of the head a wet or dry matted appearance with isolated brownish plaques and stains. Some birds develop submaxillary edema. Many birds show open mouth breathing, dyspnea, altered vocal sounds, reduced intake of feed and water, and reduced activity. These signs diminish after 2 to 4 weeks of infection (38,84,165), but the birds may become more susceptible to other infectious agents.

The gross lesions in the upper respiratory tract are characterized by generalized softening and distortion of the tracheal cartilage rings leading to dorso-ventral collapse (11,187). The combination of tracheal collapse and accumulation of exudates seriously impinge on the tracheal lumen.

The histopathologic lesions of bordetellosis are characterized by cilia-associated bacterial colonies, loss of cilia, and desquamation of ciliated epithelial cells (11,12). The bacterial colonies are prominent features on the cilia 1 to 2 weeks after onset of clinical signs (11,99). Squamous metaplasia of tracheal epithelium is not a feature in the early stages of infection though it may occur at a later stage. Dysplastic changes of epithelium have been recognized in birds infected for 3 to 4 weeks. Goblet cells are depleted of mucus granules from the first to the third week.
after onset of clinical symptoms. The lamina propria of the trachea contains multiple aggregates of heterophils which are replaced by lymphocytes and plasma cells with time (11,12). The plasma cells increase in numbers by the 3rd to 5th week of disease and lymphoid nodules develop in the submucosa. BALT appears hypertrophic and lymphoid nodules protrude into the bronchial lumen during the course of the infection (187).

Immunity and Vaccination

Humoral Immunity

Antibodies are produced in most turkeys infected with B. avium (11,99). Microtiter agglutination and ELISA have been used to detect serum antibodies within 2 weeks after the beginning of infection. Peak levels of antibody occur after 3 to 4 weeks of infection (11,99). Clinical signs of the disease subside within 1 week after peak antibody titers. The presence of maternal immunoglobulins against B. avium also suggests an important role for humoral immunity (13,91). Arp and Hellwig successfully inhibited the adherence of B. avium to the tracheal mucosa by treatment with convalescent serum and tracheal secretions from infected turkeys (87). This inhibition was possible both with local and parenteral administration of convalescent serum. The same authors, using Western immunoblots, identified at least eight different bacterial proteins recognized by antibody in serum and tracheal secretions during a 4 week course of infection (87). The serum antibody response has been shown to depend on the dosage of inoculum, age of the bird and factors influencing
colonization of the bacteria (15,42,89,92,94,100,104). Immunization studies using vaccine strains show that birds less than 3 weeks of age develop a poor immune response (92,100). Studies on the effect of *B. avium* infection on cellular immunity identified no effect on CMI (127-129), though one study by Simmons et al. (171) showed thymic lesions and suppression of lymphocyte blastogenesis. Studies on susceptibility of *B. avium* infected birds to live *Pasteurella multocida* and hemorrhagic enteritis vaccines showed that these birds have reduced immunity to these two agents.

**Vaccination**

The two vaccines that have been used for protection against bordetellosis include a live temperature sensitive mutant (ts) of *B. avium* (ART-Vax™, American Scientific Laboratories, Madison, WI) and a whole-cell bacterin (ADJUVAC-ART, Ceva Laboratories, Inc., Overland Park, KS). One more whole cell *Bordetella avium* bacterin is currently being used (Tri Bio Laboratories, Inc., Collegepark, PA). Original studies indicated that the live ts mutant vaccine could induce moderate serum antibody titers (41). However, further studies produced conflicting reports of its protective capability against the disease (89,92,94,100). Although the ts-mutant was able to adhere to respiratory epithelium and replicate slowly, it colonized poorly and induced limited immunity (14,15). The ts-mutant failed to prevent infection in day-old poults, but prevented the disease when used in poults 3 weeks of age or older. This may be due to clearance of the inoculum by maternal antibody or to the
inherent inability of younger turkeys to mount a successful immune response or the inherent resistance of 3-week old birds to resist infection.

Passive immunization with convalescent serum in 3-week old birds reduced the adherence of *B. avium* to the tracheal mucosa (14). Also, vaccination with purified pili from *B. avium* protected the turkeys against *B. avium* infection by preventing colonization (3).

It is inferred from these different studies that, the development of an effective vaccine against *B. avium* requires a better understanding of the immune responses, both maternal and acquired mucosal responses in turkeys, and better characterization of the bacterial antigens that are important in adhesion and colonization.

**Diagnosis**

Diagnosis of bordetellosis is based on clinical signs and lesions, isolation of *B. avium*, and serologic tests like ELISA, microagglutination test, biochemical tests and colony characteristics or a combination of these. Recently, a monoclonal antibody based latex bead agglutination test has been developed that can identify *B. avium* and differentiate *B. avium* from various *B. avium*-like strains (175).

**Use of Serology for Diagnosis**

Serological methods for the diagnosis of bordetellosis in turkeys have been previously described (20,93,99). An understanding of the levels of various antibody isotypes is helpful in assessing the stage of infection and the immune status in the
bird. A knowledge about the antibody isotypes in serum and secretions from mucosal surfaces could be helpful in employing easy methods of detecting antibodies. A knowledge of the serology will also help in studying immune responses to various vaccine preparations and assessing the value of each of these preparations in protecting the bird from bordetellosis.
MUCOSAL AND SYSTEMIC HUMORAL IMMUNE RESPONSE TO BORDETELLA AVIUM IN EXPERIMENTALLY INFECTED TURKEYS


P. Suresh, L. H. Arp, and E. L. Huffman

Summary

Antibody response to Bordetella avium was measured in serum and mucosal secretions of experimentally infected turkeys. One-day-old turkeys were inoculated with B. avium, and four inoculated turkeys and four uninoculated control birds per trial were euthanatized weekly from 1 through 8 weeks postinoculation (Weeks PI). Maternal antibody of the IgG isotype, present in all 1-day-old birds sampled, decreased to background levels by 3 weeks of age. Antibody (IgG, IgM, IgA) was detected in serum, tracheal washings, and lacrimal secretions in response to B. avium infection. Regardless of the sample site and isotype, antibody levels peaked at 4-6 weeks PI and then decreased rapidly from 6 to 8 weeks PI. In general, IgM and IgA peaked earlier (4-5 weeks PI) but declined more rapidly than IgG levels. Numbers of B. avium in the trachea peaked at 2-3 weeks PI and then decreased rapidly from 4 to 8 weeks PI. Even though no direct causal relationship could be determined, the results indicate that an increasing level of antibody in serum, tracheal washings, and lacrimal secretions is temporally associated with clearance of B. avium from the trachea.
Introduction

*Bordetella avium* is a gram-negative, non-fermenting bacterium that causes a highly contagious upper-respiratory-tract infection in young turkeys (8,12,14). The clinical disease is characterized by oculonasal discharge, sneezing, dyspnea, tracheal collapse, and a reduced rate of weight gain (6,13). Cilia-associated bacterial colonies, progressive loss of ciliated epithelium, and depletion of mucus from goblet cells are distinctive characteristics of bordetellosis in turkeys (2,7). Colonization of the ciliated epithelium of the upper respiratory tract appears to be of singular importance in development of the disease.

Most turkeys develop a serum antibody response to infection with *B. avium* (2,11). Serum antibodies, detected by microtiter agglutination, appear within 2 weeks after experimental exposure to *B. avium* and reach peak levels by 3 to 4 weeks postexposure (2,11). In one study, a rising serum antibody titer was associated with decreasing bacterial numbers in the trachea and recovery from clinical disease (11). The serum antibody response, combined with evidence of maternal immunity, suggests an important role for humoral immunity in prevention of and recovery from infection with *B. avium* (5,9). Convalescent serum and tracheal secretions from turkeys infected with *B. avium* inhibit adherence of the bacteria to the tracheal mucosa in turkeys (3). In addition, adherence of *B. avium* is inhibited, whether convalescent serum is administered locally or parenterally.

Although several vaccination strategies have been developed for the prevention of bordetellosis in turkeys, our understanding of the essential components
of protective immunity remains incomplete. The present study was undertaken to characterize the mucosal and systemic humoral immune response to *B. avium* and to study the relationship between antibody levels and bacterial numbers in the trachea.

**Materials and Methods**

**Turkeys.** Straight-run broad-breasted, white Nicholas turkeys were obtained commercially (Willmar Poultry, Willmar, MN.) at 1 day of age; the turkeys used were free of *Mycoplasma meleagridis* and Newcastle disease virus (NDV). Turkeys were identified by wing-band and randomly assigned to one infected and one control group for each trial. Forty to 45 turkeys were assigned to each group to compensate for possible mortality. Turkeys in each group were placed in heated brooders (wire cages) housed in separate identical environmentally controlled rooms with ample ventilation at a temperature of 29-30 C. All turkeys were provided with antibiotic-free commercial starter (Purina Mills Inc., St. Louis, MO.) and water *ad libitum*. The birds were maintained in brooder batteries for the first 3 weeks and then moved to wire grower cages.

**Bacteria and Inocula.** *Bordetella avium* strain 75 was originally isolated in Iowa from the trachea of a young turkey with respiratory disease (2). *Bordetella avium* strain F9000336 was similarly isolated in California and provided by Dr. Richard P. Chin (Fresno, Calif.) (15). Bacteria were cultured aerobically with agitation for 24 hours in brain-heart infusion broth, and cultures were then diluted 1:100 with cold phosphate-buffered saline (PBS) to a final bacterial concentration of about $10^7$
colony-forming units (CFU) per ml. Each inoculum was maintained on ice and sampled for determination of CFU/ml immediately after inoculation. The inocula and all subsequent tracheal isolates were verified as *B. avium* by reaction in a latex bead agglutination test (15).

**Experimental Design.** Two identical inoculation trials were conducted. At 2 days of age, four turkeys from each group were selected for collection of serum, tracheal washings, and lacrimal secretions. In each trial, the remaining turkeys in one group were inoculated with *B. avium*, and turkeys of the other group served as uninoculated controls. *Bordetella avium* strain 75 was used as inoculum in the first trial and strain F9000336 was used in the second trial. Each week, four infected turkeys and four uninfected control turkeys were euthanatized for bacterial culture and for collection of serum, tracheal washings, and lacrimal secretions. Clinical signs of the disease were recorded weekly and gross lesions were recorded at necropsy. Enzyme-linked immunosorbant assay (ELISA), was used to measure levels of IgA, IgM, and IgG in serum and mucosal secretions.

**Sample collection.** Lacrimal secretions (3.5 μl) were collected from each conjunctival sac of all sampled birds by absorption into a 3-mm disc of No. 42 Whatman filter paper, which was placed under the eyelid until saturated. This procedure was standardized among birds by time and volume absorbed by the disc. Discs from each eye were combined and placed with 600 μl of diluent (0.02 M PBS with 0.05% Tween 20, pH 7.4) and stored at -70 C until required for the (ELISA). To obtain tracheal washings, birds were euthanatized by intravenous injection of
pentobarbital sodium, and the cervical trachea was aseptically exposed. The trachea was clamped midway from the larynx to the thoracic inlet, and 200 μl of diluent was instilled into the upper tracheal segment. The trachea was gently massaged for 1 minute, and tracheal washings were collected with a syringe and catheter, further diluted to a total volume of 600 μl, and stored at -70 C until required for ELISA.

For quantitative bacterial culture, a 1-cm segment of trachea was excised immediately below the clamp and placed in 9 ml of cold PBS with 1% Triton X-100 (Eastman Kodak Co., Rochester, NY.) for 1 hour. For collection of serum 2 ml of blood was collected from the jugular or brachial vein of each bird, and serum was separated.

**ELISA.** The relative quantity and isotype of antibody in serum, lacrimal secretions, and tracheal washings were determined by an ELISA modeled after that described by Hopkins et al. (10). Flat-bottomed 96-well Immulon 2 plates (Dynatech Laboratories, Chantilly, VA.) were coated with whole-cell *B. avium* strain 75, washed, and blocked with 0.02 M PBS containing 5% normal goat serum (Bethyl Laboratories, Inc., Montgomery, Texas). Plates were stored in plastic bags at 4 C and used within 3 months. Diluted tracheal washings and lacrimal secretions were thawed, mixed, and centrifuged to remove exudate and debris. Serum was mixed 1:100 in diluent. Test wells of each plate were filled with 100 μl of sample and incubated at 37 C for 1 hour. Plates were washed three times with 0.02 M PBS containing 0.1% normal goat serum, emptied, and tapped dry. Two hundred (200 μl) of horseradish peroxidase-conjugated goat anti-chicken (IgG, IgM, and IgA) antibody (Bethyl), diluted 1:800,
was added to each well as needed. Chicken antibodies used for immunization of goats were purified by affinity chromatography (Bethyl Laboratories) and evaluated by gel immunodiffusion (data not shown). Plates were incubated 2 hours at 37 C and then washed three times as before. The ABTS substrate (Kirkegaard and Perry Laboratories, Gaithersburg, Maryland) was prepared immediately before use by mixing equal volumes of solutions A and B. One-hundred microliters of substrate was added to each well, and the reaction was stopped after 9 minutes by the addition of 50 µl of 0.2 M sulfuric acid. Absorbance (A) of each well was determined at 410 nm in a MR 700 microplate reader (Dynatech). Positive control wells on each plate were loaded with pooled convalescent serum for IgG and IgM and pooled tracheal washings for IgA collected from _B. avium_-infected turkeys in previous studies. Negative control wells were loaded with diluted serum from normal turkeys not exposed to _B. avium_. Additional control wells lacked antigen, primary antibody, secondary antibody, and substrate. All samples were tested in duplicate on different plates.

**Quantitative bacterial culture.** Tracheal segments in cold PBS with Triton-X 100 were held at 4 C for 1 hour and then mixed 1 minute on a vortex mixer at the maximum setting. All of these solutions were repeatedly diluted 1:10 in cold PBS (pH 7.4) and plated immediately on blood agar as previously described (1). Plates were incubated 48 hours at 37 C, and colonies were counted.

**Statistical analysis.** The effects of treatment, bacterial strain, duplicate tests, and sample site were compared using A readings from ELISA for each of the three
immunoglobulin isotypes. Numerical data were evaluated by an analysis of variance (ANOVA) with General Linear Models (PROC-GLM) as components of the SAS software (Statistical Analysis Systems, Cary, N.C.). Split-plot analysis with treatment as the main factor and source of isotype and time intervals as the subplot factors were used to analyze the treatment effects. Significance levels of $P \leq 0.05$ were used throughout unless indicated otherwise.

Results

Turkeys inoculated with either strain of $B. avium$ developed a frothy ocular and nasal discharge, matted feathers on the head, dyspnea, sneezing, and poor weight gains from 1 to 4 weeks post-inoculation (weeks PI). Distortion and collapse of the trachea was most obvious postmortem in turkeys euthanatized 2-4 weeks PI. Clinical signs of disease and gross lesions tended to be slightly more severe in turkeys inoculated with $B. avium$ strain F9000336. Uninoculated control turkeys remained clinically normal and free from infection throughout the studies. The $B. avium$ inocula used in both the trials contained $10^7$ CFU/ml. Bacterial isolates having colony morphology suggestive of bordetella reacted positively in the latex bead agglutination test for $B. avium$. Statistical comparisons of serological and bacteriological data indicated no significant differences related to the strain of $B. avium$ used, so, data were combined for the two trials.

Fig. 1 shows $A$ values for antibody to $B. avium$ in serum, tracheal washings, and lacrimal secretions. No significant differences were seen between samples tested
Fig 1. Antibody response to *B. avium* in serum, tracheal washings, and lacrimal secretions of experimentally infected turkeys. Data are expressed as Δ from ELISA conducted from 0 to 8 weeks PI. Vertical lines on the bars indicate SEM (n = 8, combined data, two trials). The Δ readings for IgM and IgA isotypes at all locations in the control group were less than 0.1.
on duplicate plates. Because antibody was collected from the tracheal mucosa by washing, direct quantitative comparisons of antibody in serum, tracheal washings, and lacrimal secretions were not made. Therefore, all comparisons are made only with control turkeys of the same age. As all the A readings from the three collection sites for immunoglobulin isotypes IgM and IgA in the control birds were less than 0.1, no graphical presentation or significant differences are needed for comparison. A statistically significant (P ≤ 0.01) elevation in IgA and IgM was detected in serum, tracheal washings, and lacrimal secretions from 1 to 8 weeks PI. For each anatomical site, IgA reached a peak at 3-5 weeks PI and then declined through 8 weeks PI (Fig. 1). Elevation of IgM reached a peak at 5 weeks PI and declined to near baseline levels by 8 weeks PI, but was still significantly different (Fig. 1).

Maternal IgG against B. avium was detected in the serum of most turkeys sampled at 2 day of age. In turkeys of the control group, no antibody response to B. avium was detected during the study, except for maternal IgG, which went down rapidly in the first 3 weeks (Fig. 2) The IgG response to infection, first detected at 3 weeks PI, peaked at 5-6 weeks PI, and declined gradually through 8 weeks PI (Fig. 1). Numbers of B. avium colonizing the trachea increased steadily to 10^{7.1} CFU/cm at 3 weeks PI (Fig. 3) and then decreased exponentially to minimally detectable levels at 8 weeks PI in five of eight turkeys necropsied.
Fig 2. Mean IgG Absorbance (A) reading at 410nm in control group serum
(n = 8, combined data, two trials). Maternal IgG levels.
Fig 3. Colonization of the trachea by *B. avium* in trials 1 and 2. Each point represents the mean ($n = 4$) $\log_{10}$ CFUs per linear cm of trachea expressed in $\log_{10}$. 
Bacterial Count

Trial 1
Trial 2

Log_{10} CFU's/Linear cm of Trachea

Weeks Post-inoculation
Discussion

Results of these studies have suggested that antibody may play a significant role in recovery of turkeys from and immunity to bordetellosis (5,9,17). Administration of convalescent serum to turkeys inhibits adhesion of *B. avium* to the tracheal mucosa (3). In the present study, we have demonstrated that IgA, IgM, and IgG antibody isotypes are reliably produced in response to infection by *B. avium*. Additionally, these isotypes are detectable in both serum and mucosal secretions. Because *B. avium* specifically colonizes the ciliated epithelial cells of the upper respiratory tract, the quantity and quality of antibody present on the tracheal mucosa is of particular interest. Results from the present study indicate that all isotypes were present in tracheal washings, but IgA antibody may be detected in the trachea earlier than IgM and IgG. The IgM response in the trachea reached a peak at about 4-5 weeks PI, roughly the time at which numbers of *B. avium* colonizing the trachea began to decline. In contrast, IgG levels in the trachea peaked after bacterial numbers had decreased substantially. Unfortunately, results from the present study do not allow us to accurately predict the relative importance of each antibody isotype in recovery from bordetellosis.

In general, levels of IgG and IgM in the serum correlated with levels in tracheal washings and lacrimal secretions at most times during the course of infection. This finding is consistent with the idea that these isotypes are capable of moving between the blood vascular system and mucosal surfaces. Recently, transfer of IgG from the serum to lacrimal fluids has been characterized in chickens (16). In
the current study, however, it is probable that IgG detected in mucosal secretions is derived both from serum and local plasma cells. IgG was generally best detected in serum, whereas IgM and IgA were at least as readily detected in tracheal washings and lacrimal secretions.

The functional significance of anti-\textit{B. avium} antibody in the lacrimal secretions is unknown. Since the lacrimal secretions drain into the nasal cavity from the nasal lacrimal ducts, there may be some benefit in clearing bacteria from the nasal mucosa to reduce bacterial transmission and persistence. Use of lacrimal secretions for the early serologic detection of \textit{B. avium} infection is possible if one uses a detection system for either IgA or IgM antibody.

Several authors have described serologic methods for the diagnosis of bordetellosis in turkeys (4,10,11). These authors have discussed the effectiveness of ELISA and agglutination methods for detecting bordetellosis. Results of the present study indicate the importance of understanding when each antibody isotype is elevated during the course of disease. Serologic methods for detecting IgM may be most useful from the second to the fifth week of disease, whereas a method to detect IgG will aid in the diagnosis only after the first 3 weeks of infection. Our results indicate that IgG can be expected to persist in the serum beyond the eighth week of disease.

The temporal relationship between increasing serum and mucosal antibody levels and the number of \textit{B. avium} colonizing the tracheal mucosa suggests that antibody may contribute to recovery from infection. The critical time of infection
when numbers of bacteria begin to decline appears to be about 3-4 weeks after exposure. All of the isotypes measured in this study are readily detectable in the tracheal secretions during the third to fourth week of infection. Therefore, the relative importance of each antibody isotype in resistance to bordetellosis remains unknown. Additional studies will be needed to address this issue. The results of the present study may serve as a reference for those attempting to stimulate immunity to bordetellosis through either passive or active immunization.

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References


A TIME-COURSE STUDY OF THE TRANSFER OF IGG FROM BLOOD TO TRACHEAL AND LACRIMAL SECRETIONS IN YOUNG TURKEYS

Manuscript for submission to Avian Diseases.

P. Suresh and L. H. Arp

Summary

Three-week-old turkeys were injected intravenously with Bordetella avium-specific IgG and absorbance readings were measured by an ELISA at various time intervals in blood, tracheal washings and lacrimal secretions. The IgG was detected in tracheal and lacrimal secretions as early as 5 minutes after injection and reached a peak 10 minutes after injection. Thereafter, the IgG absorbance declined rapidly reaching background levels by 24 hours. The absorbance of IgG reached an approximate equilibrium in all three locations by 60 minutes. The levels of IgG in all three sites were comparable at all times from 10 minutes to 24 hours after administration. The results of this study indicate that transfer of IgG from blood to mucosal surfaces in turkeys occurs rapidly.

Introduction

The importance of antibody in the defense of mucosal surfaces is well documented in poultry. A variety of avian respiratory pathogens such as infectious bronchitis virus (IBV), Newcastle disease virus, Mycoplasma gallisepticum,
Escherichia coli and B. avium stimulate the local and systemic production of antibody (3,5,11,18). The source of antibody in respiratory secretions is from the bloodstream or from plasma cells present locally in the subepithelial tissues (7).

Of the three major antibody isotypes commonly detected in respiratory secretions of mammals, IgA is frequently predominant. In contrast, IgG is frequently the predominant isotype in respiratory secretions of avian species (2). In a quantitative study of immunoglobulin isotypes in the lacrimal secretions of chickens, Lebacq-Verhayden et al. (9) found the relative amounts of IgG, IgM, and IgA to be 0.87, 0.01 and 0.15 mg/ml, respectively. Of the three major immunoglobulin isotypes, IgG transfers most readily from the bloodstream to the mucosal surface of airways (10,12). In a recent study of the humoral immune response of young turkeys infected with B. avium, significant amounts of IgG, IgM, and IgA were measured by ELISA in tracheal washings (15). The relative amount of IgG in the serum compared closely with amounts in the tracheal and lacrimal secretions over the course of infection. A recent study by Toro et al. (17) using I^{35}-labelled chicken IgG showed similar movement of parenterally injected IgG from the bloodstream to lacrimal secretions. Studies by these authors also showed a similar kinetic pattern of IgG in both lacrimal fluid and serum in response to IBV vaccination.

Few studies have been conducted on the metabolism and passive transfer of immunoglobulins in turkeys. Dohms and Saif (1978) showed that IgG is the primary immunoglobulin transferred from hen to the egg. These authors considered the movement of IgM and IgA into the egg as negligible and unimportant (4). However,
there has been no study of IgG movement from the bloodstream to mucosal secretions in turkeys. The purpose of this study was to document movement of IgG from the bloodstream to the tracheal and lacrimal secretions in young turkeys.

Materials and Methods

Experimental Design. Young turkeys were injected intravenously with *Bordetella avium*-specific IgG and levels of specific antibody were determined in serum, lacrimal secretions, and tracheal washings at time intervals from 5 minutes to 72 hours. Thirty-three turkeys were injected with 0.9 ml of *B. avium*-specific IgG (8.7 mg/ml). Samples of blood, lacrimal secretions, and tracheal washings were collected from three turkeys at 5, 10, 20, 40, and 60 minutes, 2, 4, 8, 24, 48 and 72 hours after administration of IgG. In addition, three turkeys, that were not injected with antibody, were euthanatized and sampled at the beginning and end of the study. The samples of serum, tracheal washings and lacrimal secretions were evaluated by ELISA for *B. avium*-specific IgG.

Turkeys. Thirty-nine, one-day-old, straight run, Broad-breasted white Nicholas strain turkeys were obtained from Willmar Poultry, Willmar, MN. and maintained in brooder batteries for two weeks, and then moved to wire grower cages. Turkeys were individually identified with numbered wing-bands. Antibiotic-free commercial starter (Purina Mills Inc., St. Louis, MO.) and water were provided *ad libitum*. The turkeys used in this experiment were determined to be free of maternal antibody to *B. avium* by ELISA.
Purification of Immunoglobulin G. Several three-week-old turkeys were immunized subcutaneously with live *B. avium* twice at 2 week intervals. The blood from these turkeys was harvested one week after the last immunization and serum was separated and pooled with serum obtained from some birds used in an earlier study which had been experimentally infected with *B. avium* by intra-nasal and intra-ocular routes (16). IgG from this pooled serum was purified by a combination of Sephadex and DEAE column chromatography as previously described (13). Briefly, the pooled sera were delipidated at 4 C using 4 ml of 5% dextran sulfate and 10 ml of 0.25 M MnCl₂ per 40 ml of whole turkey serum. Precipitated lipids were removed by centrifugation at 8,500 X g for 15 minutes at 4 C. The supernatant was precipitated three times in 50% sodium sulfate in an ice bath. After each precipitation, the preparation was centrifuged at 3000 X g for 15 minutes at 4 C. The pellet was resuspended in one-half the original volume in PBS, pH 7.6. After the final step, the resuspended pellet was dialysed for 48 hours in PBS with constant stirring at 4 C. This preparation was then subjected to chromatography on a Sephadex G-200 (2.5 X 100 cm) column and fractions were collected. The fractions of the second peak were concentrated and recycled on a Sephacryl HS-200 (2.5 X 45 cm) column and all fractions from a single symmetrical peak were pooled. These concentrated fractions were then subjected to DEAE chromatography with a series of PBS buffer changes at pH 7.6, 6.4, 5.8, 5.4, 4.7 and 4.4. The IgG was eluted at 0.06 M PBS, pH 7.6 to a maximum concentration. These fractions were reconcentrated 12 fold by recycling in the same PBS on DEAE three times. Further concentration of the specific IgG was achieved by
CNBr affinity chromatography on a *B. avium*-OMP bound CNBr activated Sephadex column (1 x 30 cm) as described (8,14). Purity of the IgG solution was assessed by agar gel diffusion and ELISA. For ELISA, plates were coated with *B. avium* followed by specific IgG. The secondary antibodies used were goat anti-chicken IgG, IgM and IgA which were tested by agar gel diffusion and ELISA for specificity. The final concentration of the anti-*B. avium* IgG was 8.7 mg/ml.

**Sample collection.** At each time point, blood, tracheal washings and lacrimal secretions were collected from three turkeys. Blood (2ml) was collected from jugular or brachial vein and serum was separated. Lacrimal secretions (3.5 µl) were collected from each conjunctival sac by absorption into a 3-mm disc of No. 42 Whatman filter paper which was placed under the eyelid until saturated. This procedure was standardized among turkeys by time and volume absorbed. Discs from each eye were combined and placed with 600 µl of diluent (0.02 M PBS with 0.05% Tween 20, pH 7.4) and stored at -70 C until required for the ELISA. To obtain tracheal washings, turkeys were euthanatized by intravenous injection of pentobarbital sodium, and the cervical trachea was aseptically exposed. The trachea was clamped midway from the larynx to the thoracic inlet, and 200 µl of diluent was instilled into the upper tracheal segment. The trachea was gently massaged for 1 minute, and tracheal washings were collected with a syringe and catheter, further diluted to a total volume of 600 µl, and stored at -70 C until required for ELISA.

**ELISA.** The relative quantity of *B. avium* specific IgG in lacrimal secretions, serum and tracheal washings was determined by an enzyme-linked immunosorbent assay
modeled after that described by Hopkins et al., (6,16). Flat-bottomed 96-well Immulon 2 plates (Dynatech Laboratories, Chantilly, Virginia) were coated with whole-cell *B. avium* (strain 75), washed, and blocked with 0.02 M PBS containing 5% normal goat serum (Bethyl Laboratories, Inc., Montgomery, Texas). Plates were stored in plastic bags at 4°C and used within 3 months. Diluted tracheal washings and lacrimal secretions were thawed, mixed, and centrifuged to remove exudate and debris. Serum was mixed 1:100 in diluent. Test wells of each plate were filled with 100 µl of sample and incubated at 37°C for 1 hour. Plates were washed three times with 0.02 M PBS containing 0.1% normal goat serum, emptied, and tapped dry.

Next, 200 µl of horseradish peroxidase-conjugated goat anti-chicken IgG antibody (Bethyl), diluted 1:800, was added to each well as needed. Chicken antibodies used for immunization of goats were purified by affinity chromatography (Bethyl Laboratories, Inc., Montgomery, Texas) and evaluated by gel immunodiffusion. Plates were incubated 2 hours at 37°C and then washed three times as before. The ABTS substrate (Kirkegaard and Perry Laboratories, Gaithersburg, Maryland) was prepared according to the manufacturer's directions. One-hundred microliters of substrate was added to each well, and the reaction was stopped after 9 minutes by the addition of 50 µl of 0.2 M sulfuric acid. Absorbance (A) of each well was determined at 410 nm in a MR 700 microplate reader (Dynatech Laboratories, Chantilly, Virginia). Positive control wells on each plate were loaded with pooled convalescent serum for IgG collected from *B. avium*-infected turkeys in previous studies. Negative control wells were loaded with diluted serum from normal turkeys not exposed to *B. avium*. All
samples were tested in duplicate on different plates.

**Statistical Evaluation.** All the numerical data were evaluated by analysis of variance (ANOVA) with PROC-GLM as components of SAS software (Statistical Analysis Systems, Inst., Cary, NC). The effects of the three locations, time intervals and duplicate tests were determined by this model. The levels of significant differences between means were always expressed at $P < 0.05$, unless indicated otherwise.

**Results**

High absorbance readings of *B. avium*-specific IgG were detected in tracheal washings and lacrimal secretions 5 minutes after the intravenous administration of IgG (Fig.1). Peak readings of specific IgG in the serum were detected at 5 and 10 minutes post-administration (PA). The absorbance readings of specific IgG reached a peak in the lacrimal secretions at 5 minutes PA and in the tracheal washings at 10 minutes PA. From 10 to 40 minutes PA there was a rapid decline in specific IgG from serum and mucosal sites. The rate of antibody decline in fluids sampled was much less from 60 minutes to 48 hours PA compared with the 10 to 40 minute time period PA. By 72 hours PA, levels of antibody detectable in all fluids were similar to levels in control turkeys. Absorbance readings of specific IgG were comparable in serum, tracheal washings, and lacrimal secretions from 60 minutes throughout the duration of the study.
Fig. 1. Mean IgG absorbance at 410 nm. For each data point, \( n = 3 \) birds. Serum —△—, Tracheal washings —▼—, Lacrimal secretions —☆—.
Discussion

This study demonstrated the rapid movement of IgG from the blood to tracheal and lacrimal secretions in normal turkeys. Substantial movement occurred by the first sample time at 5 minutes PA. Our study differed from other studies on immunoglobulin kinetics in that we employed a specific ELISA system to detect the IgG in contrast to radiolabelled immunoglobulins usually employed for similar studies. In a recent study in chickens using $^{135}$-labelled IgG, a similar pattern of movement was noted from serum to lacrimal secretions (17).

The kinetics of IgG movement during the first 60 minutes PA could be due to one of several mechanisms including diffusion or active transport. We did not design our study to detect any specific mechanism of transfer of IgG. However, either of these two mechanisms could occur, as there might be specific receptor mediated mechanism favouring active transport which is highly functional or it could occur by a relatively simpler process such as diffusion. In contrast to IgM and IgA, IgG may diffuse across normal tissue barriers, since it has a relatively low molecular weight (12). The nearly parallel clearance of IgG from serum, tracheal washings, and lacrimal secretions from 10 to 40 minutes PA suggests that levels of IgG had been reached in all the three fluids. Also the rapid clearance of the purified preparation of IgG used in this study might partially be due to relatively shorter half-life (t1/2) in comparison to native IgG. We could not account for all the IgG that was injected in our study. Certainly, a significant amount of IgG could be lost through respiratory and lacrimal secretions over time, but other large mucosal surfaces such as the
gastrointestinal tract were not examined. Some loss may also be expected from the hepatobiliary and urinary systems.

If IgG has a role in bacterial clearance it would be in the initial few hours after parenteral administration. In our study, the levels of intravenously administered IgG reached baseline levels by 24 hours, which is more rapid than that reported in mammals (12). These results agree with a study by Arp and Hellwig (1), in which convalescent serum administered intravenously essentially diminished *B. avium* adherence to tracheal mucosa only during the initial 1 to 6 hours and not 24 hours after injection.

In conclusion, this study showed that IgG is readily transferred from the bloodstream to tracheal and lacrimal surfaces in healthy young turkeys. Documentation of relatively high levels of IgG in respiratory secretions in birds (2,9) and evidence of efficient movement of IgG from the blood support a possible role for IgG in defense against mucosal pathogens.

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17. Toro, H., P. Lavaud, P. Vallejos, and A. Ferreira. Transfer of IgG from serum to
This study was conducted to detect the effect of parenterally administered IgG on the colonization and clearance of *Bordetella avium* at the tracheal surface in young turkeys. In two separate experiments, three-week-old turkeys were infected with *B. avium* either after or before IgG administration. The comparisons were made between a control group which received a non-*B. avium* specific IgG (anti-KLH IgG) and the experimental group which received a specific IgG (anti-*B. avium* IgG). When given prior to bacteria, IgG reduced the numbers of colony forming units (CFUs). Apart from non-specific immune mechanisms, specific IgG also seemed to have a role in preventing colonization. Similarly, in the second experiment to study the role of IgG in bacterial clearance, when the IgG was given after bacterial inoculation, it had a significant effect in reducing the CFUs from the tracheal surface. Overall, the studies indicated a role for IgG in *B. avium* infection. These observations are further discussed in relation to the importance of IgG in such mucosal infections.
Bordetella avium is the cause of a highly contagious, upper respiratory tract disease of young turkeys (10,24). The disease is characterized by oculonasal discharge, sneezing, dyspnea, tracheal collapse, and reduced weight gain (2,23,24). The tracheal lesion is consistently characterized by colonization of ciliated respiratory epithelium by B. avium progressing to loss of ciliated cells and accumulation of mucus and exudate in the trachea (2,9). Adherence of B. avium to the ciliated epithelium is a prerequisite for colonization of the tracheal surface (2,9).

Antibodies have been shown to be important components in the defense of mucosal surfaces against viral and bacterial infections of humans and animals (18,20). Antibodies have been shown to provide protection in the respiratory tract of chickens challenged with various respiratory pathogens, such as infectious bronchitis virus, Newcastle disease virus, Mycoplasma gallisepticum, Escherichia coli, and B. avium (5,6,19). Although information regarding the concentration of various immunoglobulins in tracheal secretions is incomplete, all three immunoglobulin isotypes (IgA, IgG and IgM) have been detected in tracheal secretions (15,16,28).

Antibody in the respiratory tract may be secreted by local plasma cells or transported from the bloodstream to the respiratory mucosal surface (12). Of the three isotypes, IgG diffuses most readily from the bloodstream to the respiratory mucosa due to its relatively small size (21). Transport of IgA and IgM to the respiratory mucosal surface from the bloodstream has not been reported in avian species. In addition to the transport of antibody across normal endothelial-epithelial
barriers, antibody of any isotype may cross the endothelial-epithelial barriers during times of severe local inflammation and tissue injury.

Little is known of the role of each antibody isotype on the respiratory mucosa of avian species. In a study of the relationship between serum antibody and the adherence of *Bordetella pertussis* to human respiratory epithelial cells, the presence of IgG and IgA in the convalescent serum was directly related to the antiadhesive activity of the specific serum against the organism (30). Studies in our laboratory have shown that parenterally administered convalescent serum effectively inhibited the adherence and colonization of *B. avium* to the tracheal surface in turkeys (3). In a previous study we demonstrated the rapid and effective transfer of IgG from the bloodstream to tracheal secretions (27). Furthermore, in experimental bordetellosis of turkeys, IgG is the isotype that is present for the longest period of time compared with IgM or IgA (28). Although each of the three antibody isotypes may be important in *B. avium* infection, only IgG lends itself to the time course-study of the earliest stages of infection by *B. avium*. Preliminary studies documented that when convalescent serum (with IgG, IgM, and IgA) is administered intravenously to young turkeys, only *B. avium*-specific IgG and small amounts of IgA are detectable in the tracheal secretions (data not shown). Therefore, the purpose of this study was to determine the effect of *B. avium*-specific IgG on the colonization and clearance of *B. avium* in young turkeys.
Materials and Methods

Experimental design. In two separate experiments, 23-25 day-old turkeys were used. The first experiment was designed to study the effect of *B. avium*-specific IgG on colonization by *B. avium*. Fifty-four birds were randomly selected and divided into two groups of 27 each. Birds in the first group were injected intravenously into the wing vein with *B. avium*-specific IgG. Birds in the second group were similarly injected with a non-*B. avium* specific IgG, an IgG specific for keyhole limpet hemocyanin. Ten minutes after injection of IgG, birds were inoculated intratracheally with *B. avium* and samples were collected from 10 minutes to 48 hours.

The second experiment was designed to study the role of IgG in the clearance of *B. avium*. Fifteen turkeys were inoculated with *B. avium*. Three of these turkeys were euthanatized at 24 hours post inoculation (PI) for quantification of bacteria in the trachea and anti-*B. avium* IgG in the serum and tracheal washings. The remaining 12 turkeys were divided into two groups of 6 each. One group was injected intravenously with anti-*B. avium* IgG at 8 hour intervals and the other group was treated similarly with anti-KLH IgG at 8 hour intervals. Three birds from each group were euthanatized at 48 and 72 hours PI. Samples collected at the time of euthanasia from each turkey included: blood (serum), tracheal washings, and trachea for bacterial culture. ELISA was used to measure IgG specific for *B. avium* or KLH in the serum and tracheal washings.

Turkeys. Day-old, straight run, broad-breasted white Nicholas strain turkeys (n = 69) were obtained (Willmar Poultry, Willmar, Minn.). The birds were maintained in
brooder batteries for the first two weeks and then moved to wire grower cages. Water and antibiotic-free commercial starter feed (Purina Mills Inc., St. Louis, MO.) were provided ad libitum. All turkeys were individually identified by numbered wing bands.

Bacteria and inoculum. *Bordetella avium* strain 75 originally isolated from the trachea of a young turkey with respiratory disease was used in both experiments. Bacteria were cultured aerobically with agitation for 24 hours in brain heart infusion (BHI) broth and transferred to BHI agar plates for an additional 24 hour incubation at 37 C. The bacterial growth was washed from the plates with cold phosphate-buffered saline (PBS; pH 7.4) to make a bacterial suspension of approximately $10^{10}$ colony-forming units (CFU)/ml of inoculum. For inoculation, a calginate swab dipped in bacterial suspension was passed as gently as possible down the trachea three times. The inoculum and representative colonies from tracheal cultures were verified as *B. avium* by the latex bead agglutination test (26).

Immunoglobulin G. After inoculation of several three-week-old turkeys subcutaneously at two week intervals with live *B. avium*, blood was harvested one week after the second inoculation, serum was separated and pooled with serum obtained from some birds used in the earlier study wherein birds were experimentally infected with *B. avium* by intra-nasal and intra-ocular routes. For KLH, three-week-old turkeys were immunized subcutaneously twice at weekly intervals and blood was collected a week after the final injection and serum separated. The IgG was purified by a combination of Sephadex and DEAE column chromatography as previously
described (22). Further concentration of the specific IgG was done by CNBr affinity column (13,25). The purity was assessed by agar gel diffusion and ELISA. For ELISA, plates were coated with either \textit{B. avium} or KLH followed by specific IgG. The secondary antibodies were goat anti-chicken IgG, IgM and IgA. The final concentration of the anti-\textit{B. avium} IgG and anti-KLH IgG was 8.7 mg/ml and 9.4 mg/ml, respectively. In both experiments, turkeys were injected intravenously with 0.9 ml of concentrated anti-\textit{B. avium} IgG or with 1.2 ml of anti-KLH IgG solution.

**Sample collection.** For experiment one, serum, tracheal washings, and tracheal segments were collected from three turkeys at 0, 10, 30, and 60 minutes, and 2, 4, 8, 24 and 48 hours after bacterial inoculation. Two ml of blood was collected from the jugular or brachial vein and serum was separated. To obtain tracheal washings, birds were euthanatized by intravenous injection of pentobarbital sodium and the cervical trachea was aseptically exposed. The trachea was clamped midway from the larynx to the thoracic inlet, and 200 $\mu$L of ELISA diluent was instilled into the upper trachea. The trachea was gently massaged for one minute, and tracheal washings were collected with a syringe and catheter, further diluted in the ELISA diluent to a total volume of 600 $\mu$L, and stored at -70 C until required for ELISA. For quantitative bacterial culture, a 1-cm segment of trachea was excised immediately below the clamp and placed in 9 ml of cold PBS with 1% Triton X-100 (Eastman Kodak Co., Rochester, NY.) for 1 hour.

**ELISA.** The relative quantity of IgG specific for either KLH or \textit{B. avium} in serum and tracheal washings was determined by an enzyme-linked immunosorbent assay
modeled after that described by Hopkins et al., (11,28). Flat-bottomed 96-well Immulon 2 plates (Dynatech Laboratories, Chantilly, Virginia.) were coated with whole-cell B. avium strain 75 antigen, washed, and blocked with 0.02 M PBS containing 5% normal goat serum (Bethyl Laboratories, Inc., Montgomery, Texas). Plates were stored in plastic bags at 4 C and used within 3 months. Diluted tracheal washings and lacrimal secretions were thawed, mixed, and centrifuged to remove exudate and debris. Serum was mixed 1:100 in diluent. Test wells of each plate were filled with 100 µl of sample and incubated at 37 C for 1 hour. Plates were washed three times with 0.02 M PBS containing 0.1% normal goat serum, emptied, and tapped dry. Next, 200 µl of horseradish peroxidase-conjugated goat anti-chicken IgG antibody (Bethyl), diluted 1:800, was added to each well as needed. Chicken antibodies used for immunization of goats were purified by affinity chromatography (Bethyl Laboratories, Inc., Montgomery, Texas). Plates were incubated 2 hours at 37 C and then washed three times as before. The ABTS substrate (Kirkegaard and Perry Laboratories, Gaithersburg, Maryland.) was prepared immediately before use by mixing equal volumes of solutions A and B. One-hundred microliters of substrate was added to each well, and the reaction was stopped after 9 minutes by the addition of 50 µl of 0.2 M sulfuric acid. Absorbance (A) of each well was determined at 410 nm in a MR 700 microplate reader (Dynatech Laboratories, Chantilly, Virginia). Positive control wells on each plate were loaded with pooled convalescent serum for IgG from B. avium-infected turkeys in previous studies. Negative control wells were loaded with diluted serum from normal turkeys not exposed to B. avium. All
samples were tested in duplicate on different plates.

The ELISA method for detecting the anti-KLH IgG was similar to that described above except that each well on the plate was coated with KLH at a concentration of 1 microgram/ml and the secondary antibody goat anti-chicken IgG (HRPO) was used at 1:1000 dilution.

**Quantitative bacterial culture.** Tracheal segments, in cold PBS with Triton-X 100, were held at 4 C for 1 hour and then mixed 1 minute on a vortex mixer at the maximum setting. All of these solutions were serially diluted 1:10 in cold PBS (pH 7.4) and plated immediately on blood agar as previously described (1). Plates were incubated 48 hours at 37 C, and colonies were counted.

**Statistical analysis.** The effects of treatment and duplicate tests were compared using absorbance (A) readings from ELISA for the two locations, serum and tracheal washings. Mean CFUs were compared at various times. All numerical data were evaluated by an analysis of variance (ANOVA) with General Linear Models (PROC-GLM) as components of the SAS software (Statistical Analysis Systems, Inst., Cary, N.C.). Split-plot analysis with treatment as the main factor and time intervals and sample source as the subplot factors were used to analyze the treatment effects. Significance levels of P ≤ 0.05 were used throughout unless indicated otherwise.

**Results**

Prior treatment of turkeys with anti-*B. avium* IgG in experiment one was associated with significantly fewer CFUs of *B. avium* bacteria in the trachea at various
times PI compared to turkeys treated with anti-KLH IgG (Fig. 1). A rapid decline in
CFUs in both treatment groups from 10 to 60 minutes PI was followed by a period of
increasing CFUs from 60 minutes to 48 hours PI. Absorbance readings of IgG for
both KLH and *B. avium* decreased rapidly from 0 minutes to 48 hours PI in both
serum and tracheal washings (Fig. 2). By 24 hours PI, absorbance readings of IgG
were similar to expected background levels in both groups. Absorbance readings of
IgG between groups and between serum and tracheal washings were closely
comparable at all times.

In experiment two, there was a statistically significant (*P* \(\leq 0.05\)) difference in
CFUs of *B. avium* at 48 and 72 hours PI between the two groups (Fig. 3). In the anti-
KLH IgG treated birds, mean CFUs increased nearly two orders of magnitude (log\(_{10}\));
whereas, in the anti-*B. avium* IgG treated birds, mean CFUs remained unchanged or
slightly decreased from 24 to 72 hours PI. Absorbance readings of IgG achieved by
repeated intravenous injections were significantly greater than control absorbance
values (Fig. 4).

**Discussion**

This study indicated a possible effect of IgG on *B. avium* colonization. The
parenterally administered single dose of IgG maintained reduced CFUs at specific
times. Prevention of colonization by IgG may be explained by at least three different
mechanisms: the IgG could bind to the bacteria and enhance clearance by ciliary
flow, agglutinate bacteria and reduce the apparent CFUs, or it could prevent
Fig 1. Mean *B. avium* Log\(_{10}\) CFUs per linear cm of trachea plotted against time after inoculation. Each data point represents *n* = 3 birds. Vertical bars at each point indicate the standard deviation.
B. avium Log10 CFUs/ cm trachea
Fig 2. Mean IgG absorbance readings (A) plotted against time after *B. avium* inoculation. Each data point represents $n = 3$ birds.
Fig 3. Mean $\log_{10}$ B. avium CFUs per linear cm of trachea plotted against time after inoculation, each data point represents $n = 3$ birds. Vertical bars at each point represent the standard deviation.
Fig 4. Mean absorbance readings (A) of IgG after administration, each data point indicates n = 3 birds. Vertical bars at each point represent the standard deviation.
bacterial motility and adhesion by blocking the necessary molecules (4,7,14).

The trend in CFUs in both groups during the course of experiment one indicates the importance of non-immune clearance mechanisms. The initially rapid fall in CFUs from 10 to 60 minutes is probably due to ciliary clearance (8). At 1-2 hours PI, the CFUs of bacteria seemed to stabilize, possibly due to an equilibrium reached between the rate of ciliary clearance and the rate of bacterial replication. It is speculated that the increase in numbers from 2-48 hrs is probably due to increased bacterial replication rate or a declining effect of antibody (17).

The rapid fall in IgG absorbance readings in this experiment was expected, as seen in our earlier study and also as observed in another study (29). The trend in IgG absorbance in both serum and tracheal washings were comparable. A study by Arp and Hellwig, 1988 (3), showed that parenterally administered convalescent serum against B. avium inhibited adherence at 1 and 6 hours but not at 24 hours. This agrees with the similar effect of antibody on bacterial colonization and the associated decline of the intravenously administered IgG.

In the second experiment, the IgG absorbance was maintained at readings comparable to those seen in an experimental infection once colonization had been established. A significant difference in the CFUs between the two groups at 72 hours may indicate the effect of IgG in agglutinating and enhancing the clearance of bacteria. This effect of IgG on clearance was achieved by repeated injections of specific IgG. A single injection may not have produced a significant effect due to the rapid elimination of passively administered antibody. The CFUs in the KLH group
would probably have reached $10^6$ or $10^7$ by one week, as observed in an experimental infection.

In conclusion, this study demonstrated a possible role for IgG in both colonization and clearance of *B. avium*. However, the number of colony forming units determined in this experiment may be representing either individual or bacteria agglutinated by IgG. In vivo agglutination by IgG may lead to enhanced ciliary clearance of bacteria from the tracheal surface. In a natural infection, the initial IgM response and IgA are probably important, and if they can act along with maternal IgG, young birds may resist serious clinical disease.

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The purpose of these studies was to determine the importance of a humoral immune response in bordetellosis in turkeys. In the first study, antibodies of all three isotypes, IgG, IgA, and IgM, were measured in serum and mucosal secretions (tracheal and lacrimal) by ELISA in experimentally infected turkeys. In all three fluids sampled, the antibody levels reached a peak around 4-6 weeks post-inoculation (PI) and then decreased rapidly from 6-8 weeks PI. The IgM and IgA responses peaked earlier (4-5 weeks PI) but declined more rapidly than IgG levels. The trend of increasing antibody levels correlated with a progressive decline in *B. avium* from 4 to 8 weeks of infection following maximum numbers at 2-3 weeks of infection. This clearly supports a role for antibodies in controlling a mucosal infection such as bordetellosis. Even though no direct effect of each antibody isotype could be measured in this relationship, the results are clearly indicative of the general effect of any or all of the isotypes in clearing *B. avium* from the tracheal surface.

Of the three antibody isotypes, IgG is the isotype that persisted at all three anatomic sites. It has been shown by other researchers that IgG, at least in avian species, is present at high levels in mucosal secretions compared to other isotypes. Our pilot studies indicated effective transfer of parenterally administered IgG, but not IgM, to mucosal surfaces. Keeping all these interpretations in view, IgG seems to be an important and probably the major immunoglobulin isotype coming from the blood and passing readily to the mucosal surfaces, due to its smaller size.
Our second study effectively employed the *B. avium* specific ELISA to detect IgG transfer. This study showed that IgG given parenterally can be detected in the tracheal or lacrimal secretions in as little as 5 minutes, reaching a peak around 10 minutes after injection. The corresponding levels of IgG at the three sites had a similar trend at various time intervals. The IgG reached an almost undetectable level by 24 hours at all three sites. Studies on the transport kinetics of immunoglobulins are sparse in mammalian species and are even more rare in avian species. The IgA and IgM might use specific receptor mediated mechanisms to cross the epithelial barriers at the mucosal surface; however, IgG seems to diffuse effectively across the blood vascular and mucosal epithelial barriers. Apart from the specific mechanisms involved, the molecular form in which the immunoglobulins exist could be important as well. In birds, IgM is present as a pentamer and IgA is multimeric, existing mostly as tetramers or dimers. Hence, IgM and IgA are probably being secreted mostly by plasma cells. However, IgG secreting plasma cells are also present at local sites. Therefore, IgG might be coming from both local plasma cells and directly from the bloodstream; the latter probably being the major source. This study indirectly indicated the importance of IgG in mucosal infections in birds.

Our final study was designed to see if IgG could effectively prevent initial colonization and subsequent clearance of *B. avium* from the tracheal surface of young turkeys. The first part showed that parenterally administered IgG could inhibit *B. avium* numbers when given before bacterial inoculation, thus indicating inhibition of colonization. The mechanism of this effect on colonization can be attributed to
the ability of immunoglobulins to agglutinate the bacteria or to block adhesive molecules on the surface of the bacteria. In the second part of this experiment, repeated injections of IgG were used to maintain significant levels IgG at the tracheal surface. The *B. avium*-specific IgG caused a significant reduction in bacterial numbers during the course of the experiment at 48 and 72 hours after infection. In contrast, numbers of *B. avium* in the control group increased. Even though the reduction was not great, it is suggestive of a role for IgG in clearing the bacteria.

Thus, IgG administered parenterally could exert its effect *in vivo* in the birds. The roles of IgM and IgA on either colonization or clearance cannot be ruled out. All three isotypes might have a synergistic effect in controlling the infection. Because IgG is the maternal antibody transferred to the chick, it could be important during the initial three weeks of life. Hence, vaccination strategies that enhance maternal antibodies should be encouraged. Although, it might not be possible for IgG alone to clear an infection, especially when it is overwhelming, it may reduce clinical disease. A synergistic effect of the three immunoglobulin isotypes may be very important. Being the immunoglobulin present for the longest duration at all three anatomic sites, IgG may be useful for serologic diagnosis and evaluation of the immune status of the flock.

Overall, these experiments have demonstrated the importance of humoral immunity in avian respiratory tract infections and the transfer and protective effect of IgG in controlling the infection. These kinds of studies of humoral immune responses may be useful for the evaluation of vaccines in future experiments. The
results of these experiments could be extended to studies concerned with immunity and infection of birds with various other respiratory pathogens in the design of related vaccines and evaluation of serologic responses.

Apart from these experiments conducted to study immune responses to *B. avium* in turkeys, an attempt was made to develop a diagnostic test for detecting *B. avium*. A monoclonal antibody (IgG isotype) was developed against this bacterium and effectively employed in a latex bead agglutination test. This test clearly differentiated *B. avium* from *B. bronchiseptica* and *B. avium*-like bacteria. The latter two are frequently isolated from the trachea of turkeys and are often confused with *B. avium*. This test was found to be simple, rapid and specific in distinguishing these related bacteria and is an important tool for researchers or diagnosticians dealing with respiratory infections in turkeys.
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I thank my parents for my education and for their confidence in my progress. I am thankful to my wife, Puppy, for her tireless efforts in taking care of me and my son; to my son, Nithin, for making this experience pleasant and adding meaning to my efforts.

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Finally, I am grateful to my late uncle Sri. P. N. Ghatikachalam, for the encouragement he gave and the pride he took in my success. I thank God for making this all possible.
APPENDIX

A MONOCLONAL ANTIBODY-BASED LATEX BEAD AGGLUTINATION TEST
FOR THE DETECTION OF BORDETELLA AVIUM
A Monoclonal antibody-based latex bead agglutination test for the detection of
Bordetella avium

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SUMMARY

The purpose of this study was to develop a rapid method to distinguish *Bordetella avium* from closely related *Bordetella avium*-like and *Bordetella bronchiseptica* bacteria. A monoclonal antibody of the IgM isotype was produced in BALB/c mice against live *B. avium* strain 75. The monoclonal antibody, in the form of ascites fluid, was added to a bovine serum albumin-glycine buffer (pH 8.6) and adsorbed to 3.03-μm-diameter latex beads. Optimum concentrations of antibody, beads, and bacteria were determined. The latex bead conjugate was tested against 40 isolates of *B. avium*, 24 isolates of *B. avium*-like bacteria, 17 isolates of *B. bronchiseptica*, two isolates of *Alkaligenes faecalis*, and several other common genera. Strong agglutination occurred with all *B. avium* isolates and the 2 isolates of *A. faecalis*. Weak agglutination occurred with *Staphylococcus aureus* and two isolates of *B. bronchiseptica*. There was no agglutination with any of the *B. avium*-like isolates. The latex bead agglutination test may be useful as an aid in the identification of *B. avium* when used in conjunction with other criteria.

INTRODUCTION

*Bordetella avium* is the causative agent of avian bordetellosis, a highly contagious upper respiratory tract disease of young turkeys (3,5,11). The diagnosis of bordetellosis is currently based on characteristic clinical signs, respiratory tract lesions, and isolation of *B. avium* from the trachea (2). *B. avium* and closely related
bordetella-like species produce pearl-colored, pinpoint (< 1 mm) colonies on MacConkey's agar 24-48 hours after primary isolation from trachea. The rapid differentiation of *B. avium* from *B. bronchiseptica* and *B. avium*-like bacteria has been a diagnostic problem (3).

*B. avium* can be differentiated from *B. bronchiseptica* by the urease reaction. *B. avium* is urease negative and *B. bronchiseptica* is urease positive (4,8,12). The greatest diagnostic dilemma comes with trying to distinguish *B. avium* from *B. avium*-like bacteria (3,7). *Bordetella avium*-like bacteria are urease negative, but they are nonpathogenic and adhere poorly to the tracheal mucosa (1,7). Variations among strains and growth conditions make colony morphology an unreliable means for differentiating these closely related bacteria (3,6). The capacity of *B. avium* to cause hemagglutination of guinea pig erythrocytes provides a reliable method to distinguish between *B. avium* and *B. avium*-like bacteria, but the test is inconvenient and time-consuming for many laboratories (2,7). MAb and latex bead agglutination tests have been used effectively to diagnose many fungal and bacterial infections. A latex agglutination test was found to be sensitive and specific for identifying 200 strains of *Escherichia coli* serotype O157:H7 (9). The purpose of the present study was to design a simple diagnostic test to distinguish *B. avium* from closely related *B. avium*-like bacteria and *B. bronchiseptica* by employing a MAb against *B. avium*.

**MATERIALS AND METHODS**

*Monoclonal antibody production.* MAbS were produced using BALB/c mice against
immunized by intraperitoneal injection with a 0.2 ml suspension containing $10^8$ colony-forming units (CFU) of B. avium/ml in phosphate-buffered saline (PBS). Mice were reinjected 3 weeks later. Three days before cell fusion, mice were reinjected intravenously. Spleen cells were harvested from the immunized mice and fused with SP2/O cells. Primary hybrids were cloned and screened by enzyme-linked immunosorbent assay (ELISA) for production of antibody to live B. avium strain 75, B. avium-like bacteria, and Bordetella bronchiseptica. Clones that produced antibody against B. avium were implanted in mice for ascites fluid production. Two MAbs were evaluated for use in a latex bead agglutination test. The isotype of each MAb was determined using an ELISA kit (Zymed Laboratories, South San Francisco, Calif.). MAbs 3H4-G8 and 3G10-D2 were determined to be IgG3 and IgM, respectively. Preliminary immunoblotting (performed by Dr. Vincent Collins, Washington University, St. Louis, Missouri) has shown that 3G10-D2 reacts strongly with a ~20,000-molecular-weight protein of B. avium. Minor cross-reactivity was also seen to outer-membrane proteins of B. bronchiseptica and B. avium-like bacteria.

**Coating of latex beads.** The adsorption technique used to coat latex beads with MAb was modified from Masson et al. (10). A 0.1% solution of bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, Missouri) was prepared in glycine buffer (GB pH 8.6) and 900 µl of the BSA-GB solution was transferred to an Eppendorf tube for mixing. The BSA-GB solution was mixed in a vortex mixer and incubated 1 minute at 37 C. MAb contained in 40 µl of mouse ascites fluid (1.97 mg protein/ml) was added to the BSA-GB solution and mixed twice while incubating for 5 minutes at 37
C. Next, 800 μl of a 2.5% (wt/vol) suspension of 3.03 μm diameter latex beads (Polysciences Inc., Warrington, Pa.) was added to the BSA-GB-ascites fluid solution, mixed on a vortex mixer for 1 minute, and then incubated 15 minutes at 37 C. The coated latex beads were stored at 4 C until used. Various incubation times, concentrations of MAbs, and storage conditions were evaluated before selecting the above method for producing a rapid, definitive reaction.

**Bacterial isolates.** A total of 40 isolates of *B. avium*, 24 isolates of *B. avium*-like bacteria, and 17 isolates of *B. bronchiseptica* were evaluated for reactivity in the latex bead agglutination test (Tables 1-3). Many of the isolates were used in previous studies by investigators throughout the United States during the past decade. All *Bordetella* spp. were evaluated for growth characteristics on MacConkey's agar, urease reaction, and capacity to agglutinate guinea pig erythrocytes to ensure proper species grouping. In addition, two American Type Culture Collection isolates of *Acaligenes faecalis* and single isolates of *Escherichia coli*, *Pseudomonas* sp., *Staphylococcus epidermidis*, *S. aureus* and *Proteus* sp., were tested. Each isolate was streaked onto brain-heart-infusion agar or MacConkey's agar and incubated 24 hours at 35 C. Isolated colonies were picked from the plates for testing. In preliminary studies, a bacterial concentration of ~10⁹-10¹⁰ (CFU)/ml was found to produce a strong immediate agglutination reaction; however, bacterial concentrations as low as 10³ CFU/ml also produced detectable reactions.

**Test procedure.** A single drop (50 μl) of PBS was placed on a black porcelain or plastic surface. A single bacterial colony was picked from solid medium and stirred
into the PBS to form a turbid suspension. A single drop (50 μl) of the coated latex bead suspension was applied and stirred into the bacterial suspension with a wooden applicator stick. The plate was rocked every 15 seconds and the agglutination reaction was observed at and after 30 seconds and 2 minutes. The agglutination reaction and time taken were scored. Typically, a 4+ reaction was observed as spontaneous large floccules forming within 30 seconds, a 3+ reaction showed visible flakes within 30 seconds, a 2+ reaction showed small flakes in between 30 seconds and 2 minutes and a 1+ reaction showed small flakes after 2 minutes. A negative reaction (-) was always characterized by a uniform suspension with no visible flakes. Various reaction volumes and incubation times were evaluated before selecting the procedures described above.

RESULTS

The latex bead agglutination test utilizing the MAb 3G10-D2 provided a rapid, simple means for distinguishing most isolates of *B. avium* from *B. avium*-like bacteria. Of the 40 bacterial isolates identified as *B. avium* based on growth on MacConkey’s agar, typical colony morphology, a negative urease reaction, and the capacity to agglutinate guinea pig erythrocytes, all isolates caused an obvious and rapid agglutination reaction (Fig. 1) (Table 1). None of the *B. avium*-like bacteria produced a detectable reaction (Table 2). Of the 17 *B. bronchiseptica* isolates, two produced a agglutination reaction after 2 minutes (Table.3). Of the other bacterial species, the two isolates of *A. faecalis* produced an obvious and rapid agglutination reaction
Fig 1. Latex bead agglutination reaction. (I) A typical ++++ positive reaction to *B. avium* strain 75, (II) a ++ reaction to *Bordetella bronchiseptica* strain 1188, and (III) a negative reaction to *B. avium*-like strain 001. See Materials and Methods for explanation of scoring.
comparable to that produced by *B. avium* isolates. *S. aureus* caused a weak agglutination reaction (Table 3).

Both MAbs provided easy-to-read latex agglutination reactions with most isolates of *B. avium*. MAb 3G10-D2 gave a slight cross-reaction with two isolates of *B. bronchiseptica* and MAb 3H4-G8 cross-reacted with 4 strains of *B. bronchiseptica*. Both 3HA-G8 and 3G10-D2 cross-reacted with both strains of *A. faecalis*. MAb 3G10-D2, isotype IgM, produced a more rapid and obvious agglutination reaction compared with MAb 3H4-G8. All subsequent experiments were performed using MAb 3G10-D2. In preparation of the BSA-GB-ascites solution, 10 - 100 µl of ascites fluid was added to the BSA-GB solution. Volumes of less than 20 µl gave a delayed or indistinct reaction with *B. avium*, whereas volumes greater than 80 µl caused autoagglutination of the latex bead conjugate. The time allowed for adsorption of MAb to latex beads was varied from 3 to more than 15 minutes. Incubation for only 3-4 minutes resulted in a preparation that agglutinated poorly with *B. avium*, whereas incubations of ≥ 15 minutes provided satisfactory results.

The stability of the MAb-coated latex bead suspension was evaluated at 4 °C and 37 °C. The addition of 0.1% sodium azide had no effect on the agglutination reaction and therefore was used for all stability studies. When stored at 4 °C, the latex bead suspension was stable and worked normally for at least 4 months. When stored at 37 °C, the latex bead suspension had reduced agglutinating activity after 72 hours.
Table 1. Agglutination reactions of *Bordetella avium* with MAb 3G10D2-coated latex beads.

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*Note: Refer to Materials and Methods for scoring agglutination reaction.*
Table 2. Agglutination reactions of *Bordetella avium*-like bacteria with MAb 3G10D2-coated latex beads.

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^ Note: Refer to Materials and Methods for scoring agglutination reaction.
Table 3. Agglutination reactions of *Bordetella bronchiseptica* and other bacteria with MAb 3G10D2-coated latex beads.

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<th>Isolate identification</th>
<th>Source</th>
<th>State of origin</th>
<th>Year</th>
<th>Agglutination reaction^</th>
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Other bacteria:

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<th>Agglutination reaction^</th>
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<td><em>Staphylococcus aureus</em></td>
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<td></td>
<td><em>Proteus sp.</em></td>
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^ Note: Refer to Materials and Methods for scoring agglutination reaction.
The MAb-based latex bead agglutination test proved to be useful for differentiating B. avium from other closely related bacteria. Adsorption of the MAb to latex beads was not technically difficult, and the reaction was acceptable over a wide range of concentrations of MAb, latex beads, and bacteria. The test is best suited for distinguishing colonies of B. avium from closely related bacteria on primary cultures from turkey tracheas. The latex bead agglutination test meets the minimum criteria established for the project.

Minor cross-reactivity with two isolates of B. bronchiseptica was not unexpected, as a previous study showed antigenic relatedness among the Bordetella spp. (8). Unfortunately, the cross-reactivity with B. bronchiseptica was not detected when primary hybrids were screened by ELISA. Although most isolates of B. avium produced a much more pronounced agglutination reaction compared with B. bronchiseptica, the urease reaction should be used to support the diagnosis. The strong cross-reactivity of the MAb to two isolates of A. faecalis is more of a curiosity than a problem. We did not isolate A. faecalis from the tracheas of turkeys; however, the test emphasizes the close antigenic relationship between B. avium and A. faecalis. Colonies of A. faecalis produced a green hue on blood agar plates, but they were indistinguishable from B. avium on MacConkey's agar. The reaction with S. aureus was possibly mediated by the nonspecific binding of surface protein A with the Fc portion of the MAb. Staphylococcus spp. are readily differentiated from B. avium by colony morphology.
ACKNOWLEDGMENTS

We thank Dr. Richard Van Deusen, Iowa State University Hybridoma Service, for his supervision of MAb production. We acknowledge the technical assistance of Elise Huffman and the assistance of Dr. Mark Evans and Darl Pringle in the screening and selection of MAb hybrids for use in subsequent studies.

REFERENCES


