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Avian Gut-Associated Lymphoid Tissues and Intestinal Immune Responses to *Eimeria* Parasites

HYUN S. LILLEHOJ* AND JAMES M. TROUT

Immunology and Disease Resistance Laboratory, Livestock and Poultry Sciences Institute, USDA Agricultural Research Service, Beltsville, Maryland 20705

INTRODUCTION

Host responses to parasitic infections are complex and involve many facets of cellular as well as humoral immune mechanisms. Many intestinal infections in poultry, such as coccidiosis, salmonellosis, and cryptosporidiosis, represent economically important diseases for the poultry industry (reviewed in reference 58). Since it is crucial to understand the intestinal immune system in order to develop any immunological control strategy against these intestinal diseases, this review will summarize our current understanding of the avian intestinal immune system and mucosal immune responses to *Eimeria* species and will provide a conceptual overview of the complex molecular and cellular events involved in intestinal immune responses to enteric pathogens.

Coccidiosis is caused by intracellular protozoan parasites belonging to several different species of *Eimeria*. Coccidia seriously impair the growth of and feed utilization by livestock and poultry. The Eimeriid coccidia exhibit a complex life cycle comprising stages both inside and outside of the host. During the in-host stage, there are both intracellular and extracellular stages and both asexual and sexual reproduction. In view of the complex life cycle of the coccidia, it should not be surprising that host immune responses to these parasites are also complex. Following coccidial infection, both antibody and cell-mediated immune responses are activated, although cell-mediated immunity plays a major role in disease resistance (reviewed in references 67 and 68). Unlike many protozoan parasites, the primary target tissue for coccidia is the intestinal epithelium. Thus, understanding the immune system-parasite interactions in the gut, leading to parasite elimination, is crucial for the design of new approaches to coccidiosis control.

The abbreviations used in this review are as follows: APC, antigen-presenting cell(s); Cs-A, cyclosporin A; CT, cecal tonsils; GALT, gut-associated lymphoid tissues; IEL, intraepithelial lymphocytes; IFN, interferon; Ig, immunoglobulin; LP, lamina propria; MAb, monoclonal antibody; MALT, mucosa-associated lymphoid tissues; MHC, major histocompatibility complex; NALT, nasal-associated lymphoid tissue; NK, natural killer; p.i., postinfection; PP, Peyer's patch; TCR, T-cell receptor; TNF, tumor necrosis factor.

GASTROINTESTINAL IMMUNE SYSTEM

In chickens, as in mammals, there exists a separate mucosal immune system that exhibits a number of unique features (reviewed in reference 58). The GALT represent a component of the MALT, which also include the bronchial, salivary, nasopharyngeal, and genitourinary lymphoid tissues. The MALT have evolved with specialized features that reflect their role as the first line of defense on mucosal surfaces. These include the presence of APC, immunoregulatory cells, and effector cell types distinct from their counterparts in the systemic immune system. Because of the unique location of the MALT and their constant exposure to environmental antigens, investigation of the mucosal immune system is crucial to our understanding of food allergy, tolerance to ingested antigens, and immune response to intestinal infections.

Following oral administration of foreign antigens, activation of helper T lymphocytes and IgA precursor B cells in GALT, especially in the PP, occurs, and these cells migrate to mucosal effector sites such as the LP of the intestine and to the upper respiratory tract to mediate antigen-specific secretory IgA antibody responses. This activation of B and T cells in the GALT

* Corresponding author. Phone: (301) 504-8771. Fax: (301) 504-5306. Electronic mail address: HLilleho@ggpl.ARSUSDA.gov.

followed by their migration to effector sites for the development of mucosal immune responses is termed the common mucosal immune system (72). The common mucosal immune system consists of two separate but interconnected compartments; there are mucosal inductive sites, which include the NALT and GALT strategically located where they encounter environmental antigens, and mucosal effector sites, which include the LP of the intestine and the upper respiratory tract (reviewed in reference 72). These mucosal effector tissues consist mainly of T cells, predominantly $CD4^+$ memory/effector T cells, but they also contain a large number of B cells and plasma cells, mainly of the IgA isotype. One of the salient features of the mucosal immune system is the intraepithelial transport of polymeric IgA into the external secretions. External transport of secretory Igs from blood or tissue fluids into external mucosae of the alimentary, respiratory, genitourinary, and nasolacrimal tracts contributes a major source of Igs to the intestine.

Gut-Associated Lymphoid Tissues

The GALT in chickens include organized lymphoid structures such as the bursa of Fabricius, CT, PP, Meckel's diverticulum, and lymphocyte aggregates scattered along the intraepithelium and LP of the gastrointestinal tract. The bursa of Fabricius, a hollow oval sac located dorsally to the cloaca, is the central lymphoid organ for B-cell lymphopoiesis and lymphocyte maturation, where antibody diversity is generated (reviewed in reference 80). The CT are discrete lymphoid nodules located at the proximal ends of the ceca near the ileocolonic junction (7). The CT are similar to the PP in structure, containing central crypts, diffuse lymphoid tissues, and germinal centers (31). In the CT, both T and B cells are present in the germinal centers, as are plasma cells expressing surface IgM, IgG, and IgA (42). The function of the CT is unknown, but active uptake of orally administered carbon particles has been shown, suggesting a role in antigen sampling (7).

The PP are lymphoid aggregates in the intestine which possess a morphologically distinct lymphoepithelium, with microfold (M) cells, follicles, a B-cell-dependent subepithelial zone, a T-cell-dependent central zone, and no goblet cells (7, 76). The PP represent the major inductive site for IgA responses to pathogenic microorganisms and ingested antigens in the gastrointestinal tract. In mammals, PP contain approximately 40% B lymphocytes, a large percentage of which bear surface IgA (47). Forty percent of the PP cells are regulatory T cells and include 40 to 50% $Lyt\text{-}1^+$ inducer/helper cells and 15 to 20% $Lyt\text{-}2^+$ cytotoxic/suppressor cells. A significant percentage of PP T cells bear Fc receptors for IgA and are important in regulation of IgA isotype responses. The PP also contain accessory cells such as macrophages (5 to 9%), functional dendritic cells, and unique phagocytic cells such as M cells, which possess numerous vacuoles reflecting active pinocytosis (10). The GALT, largely represented by PP, are an important site of IgA synthesis in mammals and contain a large subpopulation of B lymphocytes that are committed to IgA secretion (46). Recent studies showed that oral administration of antigen leads to the appearance of clonal precursors to B cells that eventually secrete primarily IgA (47). Moreover, such precursors were found to be more numerous in mucosal tissues than in the spleen and more numerous in conventional animals than in germ-free animals (71). The secretory IgA antibody is produced by plasma cells in the GALT and selectively transported through epithelial cells into external secretions. Following immunization with gut luminal antigens, antigen-sensitized, IgA-committed B cells and T cells leave the PP via efferent lymphatics, pass through mesenteric lymph nodes, and enter the bloodstream through the thoracic duct. From the blood, IgA-committed B cells migrate to and selectively localize in distant mucosal tissues, e.g., mammary, salivary, and lacrimal glands, and the LP regions of the gastrointestinal and the upper respiratory tracts, where they differentiate into plasma cells.

In contrast to PP, CT lymphocytes consist mainly of surface IgG^+ and surface IgM^+ B cells. A relatively small number of surface IgA^+ B cells were found in the CT (7). The PP and CT of chickens were easily identified at 10 days post-hatching (46). As the birds aged, the intestinal lymphoid aggregates underwent involution, such that by 20 weeks, the lymphoid follicles became less distinct and fewer in number, and there appeared to be a relative depopulation of the subepithelial zone in both the CT and PP. Not only did the morphologic characteristics of the PP vary with age, but also their abundance and distribution changed. The PP were not evident at hatching but could be identified in the intestine by day 1 or 2 and increased to a maximum of five at 16 weeks of age. Their number was then decreased by morphological involution, and at 52 and 58 weeks of age, only a single PP was evident. In adult chickens, only one PP is consistently found; it is located in the ileum anterior to the ileocecal junction. Meckel's diverticulum is a remnant of the yolk on the small intestine and usually persists as a discrete structure for the lifetime of the chickens. The exact function of this lymphoepithelial structure is not known, but it contains germinal centers with B cells and macrophages (7).

Within the gastrointestinal mucosa, there are two anatomic compartments containing immune system cells: the epithelium and the LP, which are morphologically separated by a basement membrane. The leukocytes located in the epithelium are mainly T cells and, to a lesser extent, non-T, non-B cells, whereas leukocytes in the LP are relatively enriched with immunoglobulin-producing B cells (4, 22, 26, 32). The intestinal leukocytes from chickens contain 80% lymphocytes, 10 to 15% monocytes, approximately 5% other mononuclear cells, and less than 1% polymorphonuclear leukocytes and plasma cells (7). Mechanical scraping of the muscularis mucosa of the small intestine reveals that LP leukocytes in this location consist of 80% lymphocytes, 20% monocytes, and less than 1% polymorphonuclear leukocytes. Some mononuclear cells isolated from the epithelium and LP were reported to be Ig positive, with the percentage being higher among LP lymphocytes (29.5%) than IEL (7.9%) or spleen lymphocytes (19.4%).

The localization of various lymphocyte subpopulations in the duodenum of adult chickens was examined by immunofluorescent staining with various MAbs which detect chicken lymphocyte subpopulations (20, 21, 65) (Fig. 1). Lymphocytes are highly concentrated in the LP and loosely scattered in the subepithelium and in the epithelium (Fig. 1A). Surface IgA^+ plasma cells are seen mostly in the LP (Fig. 1B). Intense staining was also observed on the outer epithelial membrane, indicating active transport of secretory IgA into external secretions. MHC class I antigen expression is evident on all cells in the intestine (Fig. 1C), whereas MHC class II antigen-expressing cells, presumably B cells and macrophages, are located mainly in the LP (Fig. 1D). No staining was observed with anti-MHC class IV MAb (not shown). $TCR\alpha\beta^+$ cells are present in both the intraepithelium and the LP (Fig. 1E), whereas $TCR\gamma\delta^+$ cells are present mostly in the epithelium (Fig. 1F). $CD8^+$ cells are located and scattered in both the epithelium and the LP (Fig. 1G), whereas $CD4^+$ cells are located mostly in the LP (Fig. 1H).

The phenotyping of intestinal intraepithelial T cells in chickens has revealed that molecular complexes similar to human and murine CD3, CD4, and CD8 antigens exist (18, 19, 65).

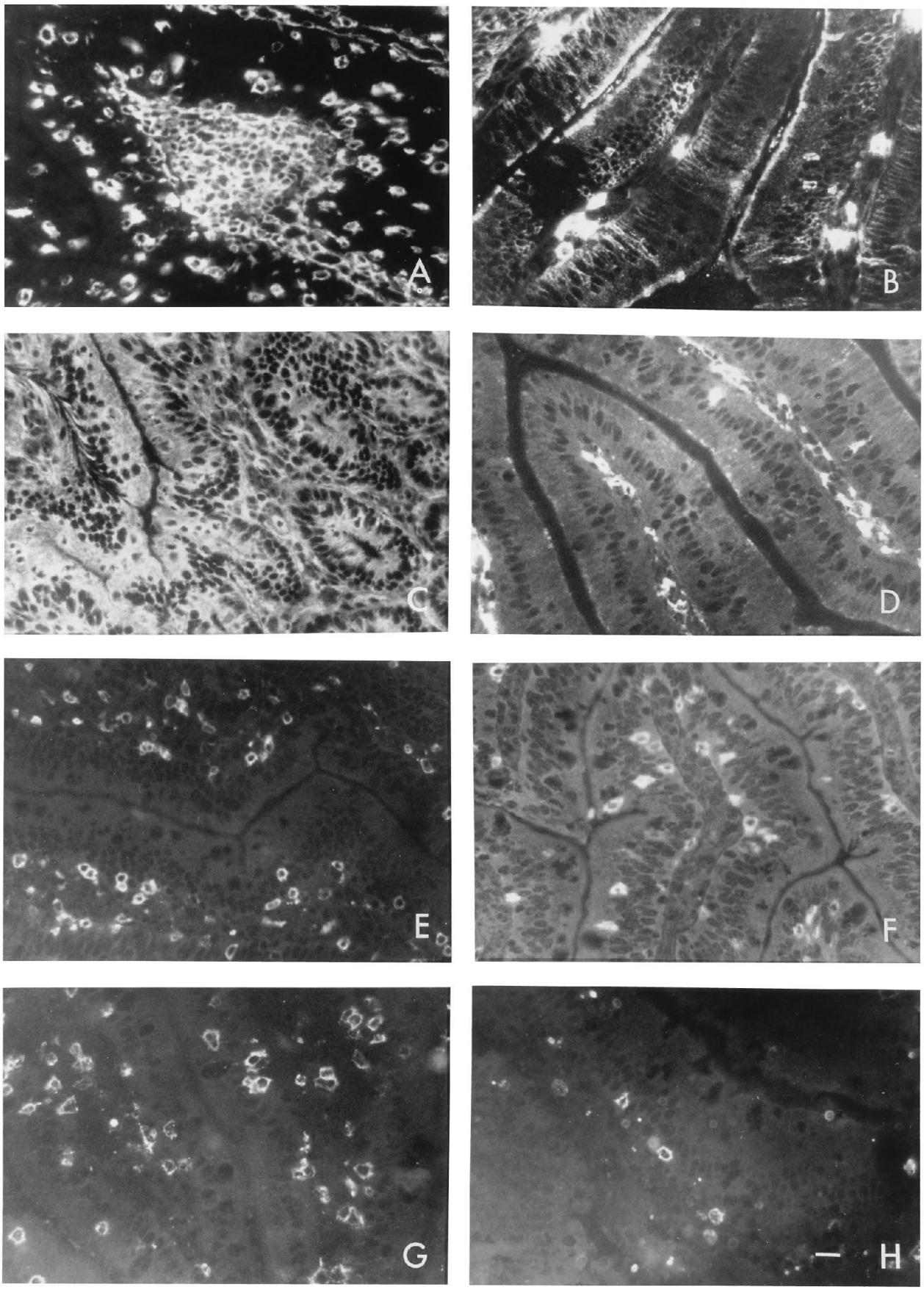

FIG. 1. Immunofluorescent staining of normal duodenum with mouse MAbs detecting lymphocytes (A), IgA (B), MHC class I (C), MHC class II (D), TCRαβ (E), TCRγδ (F), CD8 (G), and CD4 (H). Bar, 10 mm (with 40× objective).

The predominant subset of intraepithelial T lymphocytes expresses the CD3 polypeptides (gamma, delta, epsilon, and zeta) noncovalently associated with the γδ chain receptor heterodimer of the antigen-specific TCR (14). Another subset of T lymphocytes expresses the CD3 polypeptide chains in association with the αβ chain receptor. The ontogeny of T cells that bear different TCRs has been studied (19, 111). Early in fetal development, precursor thymocytes pass through the thymus and acquire distinct functions. TCRγδ is expressed on a small percentage of thymocytes by day 11 of embryonic development increases to 30% of thymocytes by day 15, and then declines to about 5% of the cells by the time of hatching. Thymocytes bearing TCRαβ and CD3 appear after 15 days of embryonic development and then quickly increase in number to exceed the level of TCRγδ$^+$ lymphocytes. Among the 85% of blood T cells that are CD3$^+$, 16% are TCRγδ$^+$ (102). TCRγδ$^+$ cells in the blood and the thymus lack both CD4 and CD8 molecules, but approximately 75% of splenic TCRγδ$^+$ cells express the avian CD8 (14). Most of the TCRγδ$^+$ cells that are localized in the splenic sinusoids and in the intestinal epithelium express the avian CD8 homolog. In contrast, TCRαβ$^+$ cells, most of which express the avian CD4 homolog, are found primarily in the splenic periarteriolar lymphatic sheath. Post-hatch development of T-lymphocyte subpopulations in the epithelium and LP was examined by using two different strains of inbred chickens (62, 63). The changes in T-lymphocyte subpopulations in the intestine reflected age-related maturation of the GALT. The ratios of TCRγδ to TCRαβ cells in epithelium and the LP in strain SC chickens were 0.96 and 1.23, respectively, at 8 weeks and 4.29 and 2.15, respectively, at 12 weeks. The ratios of jejunal T cells that express TCRγδ to those that express TCRαβ in the epithelium gradually increased from 3.4 to 4.3 by 12 weeks after hatching in strain TK and SC chickens, respectively. Jejunal CD8$^+$ IEL increased in number gradually until the chickens were 4 to 6 weeks old and subsequently declined in number as the chickens aged. CD4$^+$ cells represented a minor subpopulation among the IEL. The percentages of TCRγδ$^+$ and TCRαβ$^+$ T cells in newborn and adult SC and TK chickens showed that the numbers of these cells in IEL did not reach adult levels until 4 to 5 weeks after hatching (62). TK chickens in general contained a substantially larger number of TCRγδ$^+$ cells than did SC chickens, which suggests a genetic influence on local T-cell development. In adult SC chickens, IEL and LP lymphocytes of the duodenum and jejunum contained similar numbers of TCRγδ$^+$ and TCRαβ$^+$ cells whereas cecal IEL and LP lymphocytes contained a larger number of TCRγδ$^+$ cells (62). These studies showed that the composition of various T-cell subpopulations in the intestine depended upon the age of the host, the regions of the gut examined, and the genetic background of the host. Germinal centers seen in the CT contained scattered TCRαβ$^+$ CD4$^+$ cells but no TCRγδ$^+$ cells (14). Neither subset is present in the intestinal mucosa at hatching, and only occasional TCRγδ$^+$ or TCRαβ$^+$ cells are seen in the intestine of 3-day-old chickens. By 6 days post-hatching, both subsets are present, and the number of these cells reaches adult levels by 1 month of age.

Chicken T cells expressing a homolog of the mammalian CD4 antigen represent 20% of spleen cells and 40 to 45% of blood leukocytes, whereas 79 to 80% of thymocytes are CD4$^+$. During ontogeny, CD4 appears on thymocytes on day 13 of embryonic development and the frequency of CD4-bearing cells increases rapidly to approximately 91% by the end of embryonic development. In peripheral lymphoid organs, CD4$^+$ cells can be observed in substantial numbers only after hatching, and by the end of the first month, adult levels have been reached. CD4$^+$ cells seem to occupy certain characteristic histological locations. They are localized mainly in the periarteriolar lymphatic tissue of the spleen and the LP of the intestine. The appearance of CD8$^+$ T cells during embryonic development of chickens is very similar to that of CD4$^+$ T cells, and by the end of embryogenesis, most thymocytes express both of these molecules. In the periphery, up to 50% of spleen cells are CD8$^+$ whereas only 15% of blood leukocytes carry this antigen. Thymic and peripheral blood T cells expressing TCRγδ are CD4$^-$CD8$^-$, whereas 70% of splenic and IEL TCRγδ$^+$ cells are CD8$^+$.

A third type of cell-mediating intestinal immunity is the NK cell. NK cells represent a class of lymphocytes that are cytotoxic to hemopoietic tumor cells and cells infected by viruses or other intracellular parasites in the absence of prior sensitization (33, 106). NK cells are non-T, non-B, nonmacrophage mononuclear cells with characteristic morphology that are capable of spontaneous cytotoxicity against a wide variety of target cells. Because NK cells lack immunological memory and the MHC restriction, their cellular lineage is debatable (33, 81). There is much confusion concerning the phenotypic characterization of human leukocytes mediating natural cytotoxicity. Although MAbs specific for NK cells have been found in some mammalian species, antigenic markers specific for chicken NK cells have not been reported. Rabbit anti-asialo-GM_1 antibody, known to be relatively specific for NK cells in many mammalian species (44, 45), stains avian IEL (57). A recent study described a unique IEL subpopulation, termed TCR0 cells, showing cytoplasmic CD3 but lacking the surface TCR/CD3 complex. These cells were detected mainly in the epithelium, where most expressed CD8 antigen (13). TCR0 cells obtained from the spleen on day 14 of embryonic development, before T-cell appearance, can be enriched in the presence of conditioned medium from concanavalin A-stimulated spleen cells and can show cytotoxicity against NK-susceptible target cells (reviewed in reference 43). NK-cell activity has been reported to be present in the intestinal IEL population of mice (23, 106), rats (29), and guinea pigs (3). In chickens, NK-cell activity has been demonstrated in the spleen (50, 100), peripheral blood (28, 52), thymus (61), bursa (61), and intestine (17). Great variability in cytotoxic potential was observed among NK cells from different lymphoid organs. Furthermore, a substantial variation in NK-cell activity was also demonstrated among different strains of chickens. NK-cell activity increased as the chickens aged, and cytotoxic potential was not fully developed until they reached 6 weeks of age (61).

Effector Functions Associated with GALT

Following infection, T and B cells initiate a series of antigen-specific and nonspecific responses involving antibodies, leukocytes, and locally produced cytokines. The roles of various components of GALT in host defense against microbial infections have been studied extensively (11). Following exposure to microbes, significant architectural changes occur, which include permeability increases, infiltration of cells, increased proliferation of crypt cells, and increased production of mucin, enzymes, and Igs. Complex interactions between lymphocytes, epithelial cells, dendritic cells, and resident macrophages are involved in both secretory Ig and mucin production during the host defense process to generate a microenvironment incompatible with pathogen survival. Specific and nonspecific factors such as secretory Igs, antimicrobial intestinal peptides, and mucin influence the colonization of microbes.

Antigen presentation. Two basic types of lymphocytes are involved in antigen-specific response: B lymphocytes that express surface Ig molecules with exquisite specificities for anti-

gens, and T lymphocytes that recognize processed antigens on APC. Upon binding of an antigen to B cells expressing surface Ig, cell division and clonal expansion ensue and Igs with identical antigen specificity are secreted from the differentiated B cells. In contrast, T cells recognize small fragments of antigens that have been processed by APC only in conjunction with gene products encoded by self-MHC genes. Recent studies have provided direct molecular evidence that antigenic peptides derived from protein antigens bind to MHC class I molecules (9). The interaction of the TCR-CD3 complex with self-MHC class I and II molecules and bound peptide antigen induces a cascade of events resulting in T-cell activation (2).

Immunization with viable or nonviable antigens through the gut induces the production of local antibody and of cellular responses. The nature of the antigen influences the mode of antigen uptake, processing, and presentation and the type of APC. Dendritic cells, macrophages, and epithelial cells are representative of APCs in the gut. In addition, the M cells, a unique cell type associated with the PP, are actively pinocytic and phagocytic for both soluble and particulate (e.g., viruses and bacteria) antigens in the lumen of the gut (10). The M cells can present antigen to underlying lymphoreticular cells, leading to the sensitization of lymphoid cells present in distinct T- and B-cell zones in the PP.

Antibody-mediated immune mechanisms. In chickens, IgA and IgM are the predominant Igs in the external intestinal secretions. Although IgG is found in the gut, it is believed to be derived from the circulation or leaked from the lymphatics following permeability changes that occur during infection. Secretory IgM, which is pentameric, is effective in elimination of microbes. However, several distinctive features are important for IgA to function as a secretory antibody. One is the ability of the IgA monomer to polymerize. Another property of secretory IgA is its ability to associate with a 15-kDa peptide joining (J) chain and a 70-kDa protein, the secretory component produced by epithelial cells. The IgA-secretory component complex is internalized in endocytic vesicles, transported across the cytoplasm, and exocytosed on to the external surface of the epithelium. A functional homolog of mammalian secretory component has been described in chickens (77). A minor source of IgA in secretions is derived from blood via the hepatobiliary IgA transport system. In contrast to the transepithelial IgA pathway, hepatocytes express a specific receptor for blood IgA (2). Polymeric IgA injected into the blood was cleared into the bile in less than 3 h (96).

The major functions of secretory IgA include prevention of environmental antigen influx into internal body compartments, neutralization of viruses and microbial toxins, and prevention of adherence and colonization of mucosal surfaces by microbial pathogens. Secretory antibodies may bind to the surface of the pathogen and prevent binding to the epithelium by direct blocking, by steric hindrance, by induction of conformational changes, or by reduction of motility. In this manner, microorganisms would be susceptible to the natural cleaning functions of the mucosae. The role of secretory Igs has been documented in agammaglobulinemic patients. However, the role of secretory Igs is less clear in some poultry infections. Despite the absence of Igs, agammaglobulinemic chickens are resistant to reinfection with coccidia (55) and to leukocytozoan parasites (38). Therefore, although the primary role of sIgA is to prevent invasion of microbes into the intestine, it is less certain whether sIgA limits the course of major infections once they are established.

Cell-mediated immune mechanisms. Cell-mediated immune responses include both antigen-specific and non-antigen-specific activation of various cell populations including T lymphocytes, NK cells, and macrophages. T lymphocytes are composed of two functionally distinct subpopulations distinguishable by their surface phenotypes. Cytotoxic T lymphocytes ($CD8^+$) recognize foreign antigens in the context of MHC class I molecules, whereas helper T cells ($CD4^+$) recognize antigens in association with MHC class II molecules. Although cytotoxic T lymphocytes activity has been demonstrated in the intestine of mammals, MHC-restricted IEL cytotoxic T-lymphocyte activity has yet to be shown in chickens. There has been increased interest in the selective homing of $TCR\gamma\delta^+$ cells to the intestinal epithelium in mice (41) and in chickens (14). However, studies in humans (11) and chickens (62) showed that $TCR\alpha\beta^+$ T cells are present in intestinal IEL as well. Thus, species differences as well as other host-related factors can influence location of different T-cell populations in the gut. Great caution should be exercised in generalizing a finding that is defined in a limited situation.

Considerable interest in IEL NK cells has also developed (11). NK cells have been postulated to play an important role as a primary host defense mechanism against tumors, bacteria, and viruses, as well as in the homeostasis of normal tissues (33). It has been suggested that IEL NK cells are active in the first line of host defense because of their close proximity to the gut, where a variety of antigenic substances are introduced (17). The observation that chicken intestinal IEL contain NK cells that mediate spontaneous cytotoxicity (17) suggests that NK cells may play an important role in local defense. Intestinal IEL NK activity depends not only upon the type of target cell but also upon the incubation time and the host genetic background. Kinetic studies revealed that cytotoxicity was detectable from 2 h after incubation and progressively increased for up to 16 to 18 h. Furthermore, NK-cell activity was higher in the jejunum or ileum than in the duodenum or cecum.

INTESTINAL IMMUNE RESPONSES TO *EIMERIA* PARASITES

Life Cycles and Pathogenesis

Coccidia of the genus *Eimeria* are members of the family Eimeriidae, which belongs to the subphylum Apicomplexa. Sporulation of oocysts occurs outside the host. During this process oocysts, which are the end result of sexual reproduction, sporulate to form an oocyst containing four sporocysts, each containing two sporozoites. When sporulated oocysts are ingested by a susceptible host, the infective cycle begins. After ingestion, the sporulated oocyst undergoes excystation, which releases individual sporozoites. The sporozoites invade the intestinal epithelium and round up to form trophozoites, followed by nuclear divisions to form immature meronts (schizonts). The meronts reproduce asexually by multiple fission to form varying numbers of merozoites. The mature meronts and the host cells in which they are contained then rupture, releasing the merozoites, which undergo one to several additional generations of merogony.

Eventually, merozoites invade cells and develop into either microgamonts or macrogamonts. The former undergo multiple divisions, resulting in the formation of numerous microgametes, which are flagellated and motile. The latter give rise to a single macrogamete. Upon release, the microgametes invade cells containing macrogametes, and fertilization occurs. After fertilization, an oocyst wall forms and the oocysts mature; the host cell then ruptures, releasing the oocysts into the intestinal lumen, where they are discharged in the feces. The process of sporulation then takes place starting a new infective cycle.

The pathology of coccidial infection varies with the species

TABLE 1. Selected *Eimeria* spp. infecting mice, cattle, and chickens

Host and *Eimeria* sp.	Gut location infected
Mice	
E. falciformis	Colon, cecum
E. praegensis	Colon
E. papillata	Jejunum
E. vermiformis	Jejunum, ileum
Cattle	
E. bovis	Jejunum, ileum, cecum
E. zuernii	Jejunum, ileum, cecum
Chickens	
E. acervulina	Duodenum, upper jejunum
E. brunetti	Lower ileum, colon, proximal cecum
E. maxima	Lower duodenum, jejunum, upper ileum
E. mitis	Ileum
E. necatrix	Lower duodenum, jejunum, upper ileum (oocysts develop in cecum)
E. praecox	Duodenum, upper jejunum
E. tenella	Cecum

of *Eimeria* (Table 1) and the host, but some features are common to all infections. Clinical signs include lethargy, depression, decreased food and water intake, and a decrease in normal grooming behavior. Decreases in weight gain or actual weight loss may be apparent. The water and mucus content of fecal material is increased, and diarrhea may be present. Blood may also be present in the feces. Gross pathological lesions may include grey or white nodules or striations on the luminal surface of the intestine, and hemorrhagic enteritis may be noted. Microscopically, a pericryptal infiltrate of mononuclear cells and granulocytes is often seen, accompanied by edema and a thickening of the mucosa. In chickens, a large percentage of the cellular infiltrate is composed of $CD8^+$ lymphocytes, which are sometimes visible as large aggregates in the crypts and LP (109).

Role of Lymphocytes in Sporozoite Transport

The details of the sporozoite invasion and their subsequent translocation are not entirely defined. That *Eimeria* species tend to be very site selective suggests that sporozoites from different species recognize different host cell structures during the invasion process. Shortly after infection, *Eimeria tenella* sporozoites can be seen invading cells of the intestinal (or cecal) surface epithelium (112). Some species complete their development in the surface epithelium, while other species develop in endothelial cells of villus lacteals, the LP, or the epithelium of the crypts. Possible translocation mechanisms are discussed below.

A number of factors, including whether the host was previously exposed to the parasite and therefore has developed an immunity, influence the ability of sporozoites to invade epithelial cells. MAbs have been used to study structures associated with sporozoite invasion. MAbs that recognize surface or surface/internal antigens of the *E. tenella* sporozoite were able to inhibit invasion by *E. tenella* in vitro (5). Recently, a chicken hybridoma that secretes a MAb capable of inhibiting sporozoite invasion of lymphocytes in vitro has been developed (98). This MAb recognizes the conoid, an apical complex structure known to be involved in host cell invasion by *Toxoplasma gondii* (116). Furthermore, this MAb recognizes a common antigen shared between sporozoites and merozoites from several *Eimeria* species (97).

Limited information exists on the nature of the host receptor(s) for sporozoites. Membrane proteins of 37 and 45 kDa inhibited invasion of *E. tenella* but not *E. meleagrimitis* (5), suggesting that different *Eimeria* spp. may use different host cell structures for invasion. A MAb that purportedly recognized an epitope common to *E. tenella* sporozoites and to chicken cecal epithelial cells was developed (114). Although the significance of this shared epitope is unclear, it raises interesting questions about site selection and about a possible antigen mimicry that parasites could use to avoid host immune recognition.

The role of antibodies in inhibiting sporozoite invasion of host cells is debatable. Some studies indicate that antibodies inhibit sporozoite invasion (86, 90), while other studies do not (6, 104). When chickens immune to *E. tenella* were challenged, 50% fewer intracellular sporozoites were observed 6 h p.i. than in challenged nonimmune birds (86, 90). This inhibition of invasion may be related to specific antibodies present in the mucosa of immune birds, which act by directly blocking invasion or by enhancing intraluminal destruction of sporozoites. However, bursectomized chickens, which produce few or no antibodies, do develop protective immunity following primary infection with *E. tenella* (30, 55). Furthermore, *E. tenella* has been observed capping and shedding immune complexes (104), and sporozoites collected from the ceca of the immune birds exhibit normal development when transferred to naive chickens (85). In *E. tenella*-immune chickens, sporozoites penetrated the surface epithelium and migrated through the LP in numbers equal to those seen in naive chickens (87). However, only a few sporozoites entered the crypt epithelium and developed into trophozoites; no further development was detected. Conversely, *E. acervulina*-immune birds had 11% more intracellular sporozoites (6) than did controls, indicating that immunity does not necessarily depend on blocking invasion of sporozoites. However, since *E. acervulina*-immune birds passed no oocysts, development was arrested at some point.

Not all *Eimeria* spp. develop at the site of invasion. Some species are transported, by host cells, from the site of invasion to the site of development. Early reports suggested that macrophages (24, 112) or IEL (1, 27, 51) were responsible for sporozoite transport, although the identity of the cells was not confirmed. Although large numbers of macrophages were observed in the intraepithelial region, they only rarely contained sporozoites (51). Extracellular sporozoites were rarely seen in tissue spaces, and no developmental stages (trophozoites or meronts) were observed in IEL. Because IEL are a heterogeneous population consisting of B cells, T cells, and NK cells (62), the nature of the IEL responsible for *E. acervulina* sporozoite transport was identified by using a panel of MAbs detecting chicken leukocyte subpopulations (20, 21, 65). Following primary infection, most sporozoites were seen inside $CD8^+$ lymphocytes (108, 109), indicating that these cells are responsible for sporozoite transport. A significant number of sporozoites were also detected inside macrophages. However, macrophages are phagocytic, and it is possible that different mechanisms exist for sporozoite invasion of $CD8^+$ cells. Following secondary infection, there was an accumulation of sporozoites in $CD8^+$ cells, suggesting that sporozoites were unable to exit these cells to complete their journey to crypt epithelial cells. Furthermore, when $CD8^+$ cell-depleted chickens were infected with *E. acervulina* or *E. tenella*, there was on average a 55% decrease in oocyst production during a primary infection (110). These data further suggest a role for $CD8^+$ lymphocytes in sporozoite transport and are in agreement with observations that sporozoite transport and/or transfer from IEL to crypt enterocytes is inhibited in birds immune to *E.*

maxima or *E. tenella* (82, 90). This inhibition of sporozoite transfer to crypt epithelial cells during secondary infection could indicate that sporozoites are unable to exit once inside activated CD8 T cells. A contrasting observation suggesting no involvement of IEL in transporting *E. tenella* sporozoites (115) was recently reported. The reason for the differences observed in this study (115) and others (1, 24, 27, 51, 82, 90, 108, 109, 112) remains to be determined.

Host Immune Responses

Humoral immune responses. Animals infected with *Eimeria* spp. produce parasite-specific antibodies in both the circulation and mucosal secretions (36, 56, 74, 91). Circulating antibodies consist of IgM, IgG, and IgA (66, 107), whereas secretory IgA was detected in bile and gut washings of infected animals (66, 91). In mice, IgA^+ and IgG^+ B cells were increased in number in intestinal sections taken following *E. falciformis* infection (74). IgA^+ cells were concentrated in the upper and middle regions of the LP, while IgG^+ cells were seen primarily at the base of the mucosa (74). Although the antigen specificity of these B cells was unknown, infection clearly resulted in distributional changes.

The serum IgM response peaked at 17 days p.i. for infection with *E. tenella* (107) and at 10 to 20 days p.i. with *E. nieschulzi* in rats (91). IgG titers in serum peaked at 17 days p.i. for infection with *E. tenella* (107), 20 to 30 days p.i. for infection with *E. nieschulzi* (91), and 10 to 20 days p.i. for infection with *E. bovis* (36). Biliary sIgA was detected as early as 7 days p.i. for infection with *E. tenella* or *E. acervulina* (57) and 5 days p.i. for infection with *E. nieschulzi* (91). Challenge infection with *E. tenella* or *E. acervulina* did not elicit an anamnestic sIgA response (56). Anamnestic serum IgG responses were seen following *E. nieschulzi* challenge (91), and numbers of IgA^+ and IgG^+ cells in the intestines of *E. falciformis*-challenged mice were greater during secondary infection than during primary infection (74).

It is clear that *Eimeria* parasites do stimulate humoral immunity. However, the participation of these responses in protection against *Eimeria* parasites is unclear. Although in vitro studies showed that immune sera increase the phagocytosis of sporozoites and merozoites by macrophages in cultures (8, 75), in vivo studies involving hormonal and chemical bursectomy (30, 55) clearly indicated that antibodies play a minor role in protection against coccidiosis. It is possible that antibodies reduce the invasion of some (but not all) species of *Eimeria* or enhance the intraluminal destruction of sporozoites if parasites come into close contact with local antibodies before the parasites enter host cells. However, sporozoites and merozoites quickly enter host cells once they are inside the gut. Therefore, it is apparent that antibody-mediated responses play a minor role in protection against coccidiosis.

T-lymphocyte responses. The importance of T cells in immune responses to coccidia has been well documented (reviewed in references 67 and 68). In mice, immunity can be adoptively transferred between individuals by using spleen or mesenteric lymph node cells (95), with the latter reportedly being more effective. In chickens, both spleen cells and peripheral blood lymphocytes are capable of transferring resistance to infection to naive recipients (84).

Athymic mice and rats experience more severe primary infections and have no resistance to reinfection (83). Furthermore, a partial in vivo depletion of mouse T cells with antibody to Thy 1.2 antigen resulted in an abrogation of protective immunity to *E. falciformis* and extended the patent period (105). Similarly, depletion of $CD4^+$ cells by in vivo treatment with anti-CD4 MAb increased the severity of primary *E. vermiformis* infection but had no effect in animals with established immunity (88). In vitro depletion of $CD4^+$ but not $CD8^+$ cells obtained from immune mice prevented the transfer of immunity to *E. vermiformis* to naive mice (89). These results suggest that helper T cells may be responsible for mediating protective immunity to *E. vermiformis.*

SCID/bg mice, which lack both T and B lymphocytes, produced more oocysts than did immunocompetent mice when infected with *E. papillata* or *E. ferrisi*, two species that develop in the villus epithelium (99). However, infection of SCID/bg mice with *E. falciformis*, a species that develops in the crypt epithelium, resulted in oocyst production comparable to that in immunocompetent mice (99). These results indicate that the scope of host immunity involved in *Eimeria* infection depends upon the species of parasite as well as the site of development.

The role of T cells in coccidiosis resistance was also investigated with agents such as Cs-A, betamethasone, and dexamethasone that suppress cell-mediated immune responses. When Cs-A was given concurrently with oocysts, resistance to primary infection was enhanced (55). If Cs-A was given prior to oocyst inoculation, it increased susceptibility; if it was given prior to secondary infection, protective immunity was eliminated (55). Additionally, Cs-A treatment of chickens prior to inoculation with any of three species of turkey *Eimeria* allowed those species to complete their life cycles (48), indicating that T cells are involved in natural resistance to foreign host species. Treatment of chickens with betamethasone enhanced the severity of infection (69). Dexamethasone-treated chickens showed reduced T-cell proliferation, reduced interleukin-2 and IFN-γ production, and increased susceptibility to *Eimeria* infection (39), even though coccidium-specific IgA and IgG responses were at times enhanced. Administration of dexamethasone also allowed the development of turkey coccidia to occur in chickens (73). These results suggest a major role for T cells in mediating protective immunity to coccidian parasites in natural as well as foreign hosts.

Activation of antigen-specific T cells from *Eimeria*-immune mice, cattle, and chickens has been demonstrated by lymphoproliferation assays (35, 54). Soluble antigens from various developmental stages of *E. acervulina* or *E. tenella* induced the proliferation of T cells taken from chickens previously infected with the same *Eimeria* species (54, 85). *E. acervulina* antigens, however, induced little or no proliferation of T cells from *E. tenella*-infected chickens (54), indicating the species-specific nature of host immune responses to *Eimeria* spp. However, if very high concentrations of antigens were used, lymphocytes from *E. tenella*-immune birds responded to *E. acervulina* antigens and cells from *E. acervulina*-immune birds responded equally well to both *E. acervulina* and *E. tenella* antigens (78). These reports suggest that cross-reactive T cells exists under some circumstances. If cross-reactive T-cell epitopes do exist, the question of why cross-protection is not seen in vivo still remains.

The changes in intestinal T-cell subpopulations in the duodenum following primary and secondary *E. acervulina* infections were investigated (59). The number of duodenal IEL expressing the CD8 antigen increased in SC and TK chickens following primary infection. Following secondary infection, a significantly larger number of $CD8^+$ IEL was observed in the SC chickens, which manifested a lower level of oocyst production than did TK chickens. The $CD4^+$ IEL numbers increased 7 days after primary infection in SC and TK chickens and 7 days after challenge infection in TK chickens. Two-color immunofluorescence analysis of duodenum IEL at 10 days following challenge infection revealed that the majority of $CD8^+$

IEL coexpressed TCRαβ. Furthermore, SC chickens showed increased number of TCRαβ$^+$ CD8$^+$ cells shortly after challenge infection with *E. acervulina*. These results suggest that variations in T-cell subpopulations may reflect *Eimeria* infection-related changes in the GALT and that a significant increase in TCRαβ$^+$ CD8$^+$ IEL in SC chickens may reflect enhanced acquired immune status in SC chickens compared with TK chickens.

The growing evidence that T cells might be the primary mediators of immunity to *Eimeria* spp. led to investigations of helper T cells. In mice, depletion of CD4$^+$ cells resulted in increased oocyst production during primary infection but had little effect on resistance to challenge infection (88). This suggests that CD4$^+$ cells or the cytokines they produce are critical to the control of parasite replication during primary infections. Limited information exists concerning the role of various lymphocyte subpopulations in cytokine production during avian coccidiosis as a result of the unavailability of recombinant avian cytokines. A recent study shows that depletion of CD4$^+$ cells had no effect on primary *E. acervulina* infection but resulted in a significant increase in oocyst production following primary *E. tenella* infection (110). This difference could be related to the nature of host-parasite interaction or cell changes that occur during these infections. A large percentage of infiltrating cells were CD4$^+$ following primary *E. tenella* infection (113), while CD4$^+$ cell infiltration was not seen during primary *E. acervulina* infection (109). Such results further complicate the understanding of immunity to coccidia by suggesting that immune mechanisms may vary from one gut location to another or that different mechanisms are activated depending on the infecting *Eimeria* species.

CD8$^+$ intraepithelial cells: a dual role? As discussed above, transport of sporozoites occurs primarily in CD8$^+$ T cells. Entry into CD8$^+$ cells is apparently an active process by the sporozoites, because heat-killed sporozoites do not enter these cells and CD8$^+$ T cells are nonphagocytic (53). The reason why the sporozoites enter CD8$^+$ cells at a greater frequency than they enter other cells remains to be determined. However, CD8$^+$ cells are present in large numbers by 24 h following *Eimeria* infection (60, 109), and significantly more sporozoites are found in or next to CD8$^+$ cells in *E. acervulina*-immune chickens than in native chickens. Two-color immunofluorescence staining of the duodenum after infection with *E. acervulina* shows that at 24 h after primary infection, many sporozoites are located predominantly within CD8$^+$ T cells and macrophages (109). At 48 h after secondary infection, a significantly increased number of CD8$^+$ cells is seen in the intestine, especially in contact with parasitized epithelial cells (Fig. 2B). In contrast, fewer CD8$^+$ cells are seen in the intestine near parasitized epithelial cells at 48 h after primary infection (Fig. 2C). These findings provide evidence that suggest a protective role for CD8$^+$ IEL in *E. acervulina* infection. Depletion of either CD8$^+$ cells or TCRαβ$^+$ cells resulted in substantial increases in oocyst production following a challenge *E. acervulina* infection in chickens (110). This suggests that cells coexpressing CD8 and TCRαβ may be critical for protection against reinfection with *E. acervulina*. As opposed to primary infection, a challenge infection usually results in a reduction in the number of oocysts produced and amelioration of clinical symptoms (83). In immune hosts, sporozoites enter the gut early after infection but are prevented from developing (83); these sporozoites, when removed 24 to 48 h p.i., resume normal development upon transfer to a nonimmune host (86). Thus, acquired immunity to coccidiosis may involve immune mechanisms that both reduce the number of intracellular sporozoites and inhibit the natural progression of parasite development. Although a direct role of CD8$^+$ T cells in resistance to coccidiosis has not been proven yet, increased numbers of these cells were seen in the epithelium of the gut following secondary infection (60, 109). Furthermore, CD8$^+$ T cells were frequently seen in direct contact with parasite-infected epithelial cells in tissue sections, suggesting that infected

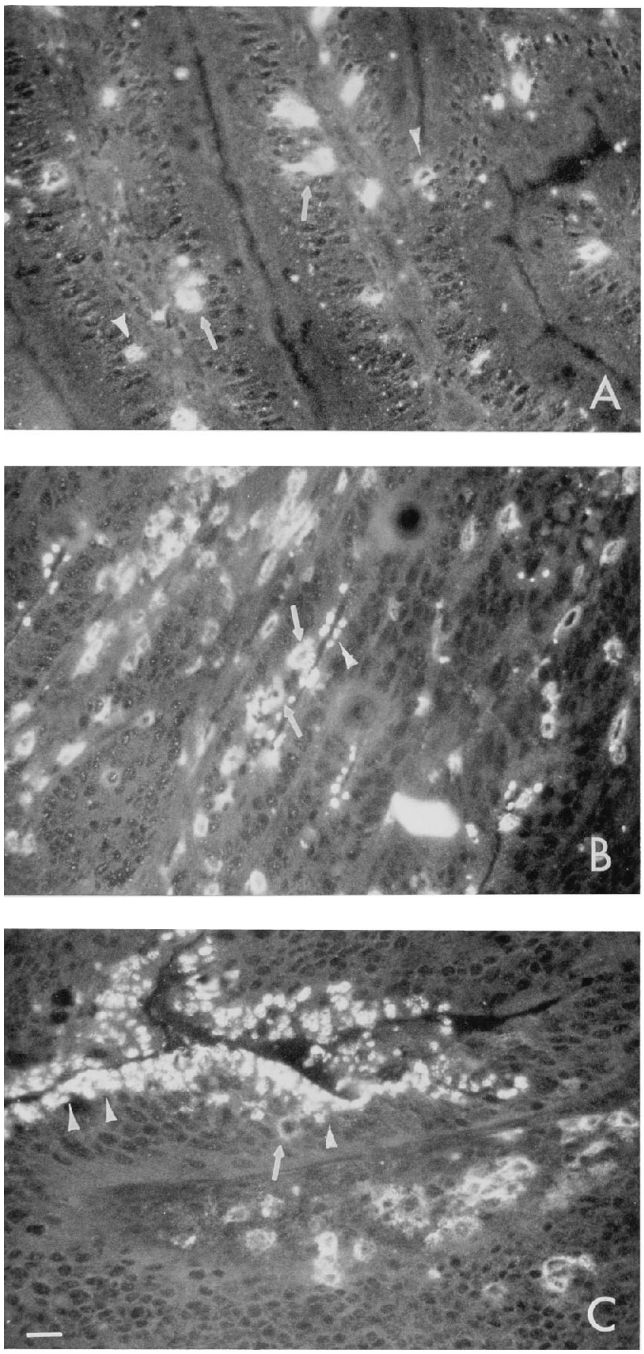

FIG. 2. Immunofluorescent staining of the duodenum after infection with *E. acervulina*. (A) Duodenum stained with anti-sporozoite and anti-CD8 MAbs 24 h after primary infection. (B) Duodenum stained with anti-sporozoite and anti-CD8 MAbs 48 h after secondary infection. (C) Duodenum stained with anti-sporozoite and anti-CD8 MAbs 48 h after primary infection. Arrowheads point to sporozoites (A) or developing schizonts (B and C), whereas arrows points to infiltrating CD8$^+$ cells. CD8$^+$ cells, especially in immune chickens, are frequently seen in close contact with parasites that are developing in the epithelium.

epithelial cells may be the target of cytotoxic T cells. An attack directed at the infected host cell would be consistent with the MHC-restricted nature of cytotoxic T cells. In mice, depletion of $CD8^+$ T cells in vivo also resulted in a slight increase in oocyst production following challenge infection with *E. vermiformis* and a significant increase in oocyst production following a challenge infection with *E. pragensis* (94). These results provide strong evidence that $CD8^+$ T cells mediate host immunity to coccidian parasites. Thus, new strategies to control coccidiosis may need to focus on manipulating the activities of $CD8^+$ lymphocytes.

Cytokines and lymphokines. Cytokines and lymphokines have been shown to influence the course of coccidial infections. Cell culture supernatant from concanavalin A-stimulated lymphocytes inhibited the replication of *Eimeria* parasites in MDBK cell cultures (64). The same supernatant, when administered to chickens, reduced oocyst production following both *E. acervulina* and *E. tenella* infections (64). Supernatants from concanavalin A-stimulated lymphocytes also inhibited the growth of *E. bovis* and *E. papillata* in bovine monocyte cultures (103) and activated murine macrophages and a bovine monocyte cell line to kill *E. bovis* parasites (34). It was also shown that chicken peripheral blood macrophages were able to transfer sporozoites to the MDBK cells in culture and that treatment with supernatants from sporozoite-stimulated lymphocyte cultures eliminated this transfer (64). The culture supernatants had no direct cytotoxic effect on sporozoites (34, 64, 103).

Various cytokines are produced by macrophages following coccidial infection. Sporozoites and merozoites of *E. tenella* induced production of a TNF-like factor by normal peripheral blood-derived macrophages in vitro (118). Treatment of chickens with anti-TNF antibody resulted in a partial abrogation of *E. tenella*-induced body weight loss in SC chickens but not in TK chickens (117). Additionally, macrophages show a biphasic production of interleukin-1 and TNF-α following *E. tenella* or *E. maxima* infection (15, 16), as well as increased production of colony stimulation factor (16). Although the exact role of TNF and interleukin-1 in coccidiosis needs to be further characterized, these findings may explain the severity of the clinical signs associated with coccidial infection. After infection with *E. acervulina*, expression of transforming growth factor β4 mRNAs increased 5- to 8-fold in intraepithelial lymphocytes and 2.5-fold in spleen cells whereas expression of the mRNAs for transforming growth factor β2 and 3 remained constant in these cells, suggesting a role for transforming growth factor β4 in *Eimeria* infection (40).

IFN-γ has been implicated in immunity to a primary *E. vermiformis* infection in mice. Depletion of endogenous IFN-γ by using a rat anti-IFN-γ MAb resulted in increased oocyst output and mortality during primary infection of mice with *E. vermiformis* (93, 94). Treatment with anti-IFN-γ MAb did not prevent the development of resistance to subsequent challenge, nor did treatment at the time of challenge disrupt immunity to the parasite. However, treatment of mice with anti-IFN-γ MAb before *E. pragensis* infection did not affect the replication of parasites following primary infection. These data suggest that the effect of IFN-γ depletion on murine coccidiosis depends on the species of parasite and the parameters of infection measured; the effect also differed in primary and secondary infections. These results, while confirming the role of IFN-γ in the resistance to some *Eimeria* infections in mice, indicate the complexity of the host immune response to coccidian parasites.

IFN production in chickens has been used as a measure of T-cell responses to coccidial antigens (15, 16, 70, 79). Lymphocytes from *Eimeria*-infected chickens produced a higher level of IFN-γ when induced with concanavalin A than did lymphocytes from uninfected chickens (70). Strain differences in *Eimeria*-induced IFN-γ production were observed (70). Treatment of MDBK, fibroblast, and epithelial cell cultures with recombinant bovine IFN-γ inhibited *E. tenella* and *E. vermiformis* development (49, 92). Pretreatment of sporozoites with IFN-γ did not affect growth, indicating that IFN-γ alters some aspects of the host cells but not those of the parasites. However, until recombinant chicken IFN-γ becomes available, the role of IFN-γ in avian coccidiosis remains to be determined.

NK cells, mast cells, and macrophages. Significant numbers of sporozoites are found within macrophages following primary and secondary infection of chickens with *E. acervulina* (109). No significant difference in the number of sporozoites inside macrophages was noted following primary and secondary infections (111). These results indicate that enhanced macrophage uptake of sporozoites in *E. acervulina*-immune chickens does not occur, although *E. tenella*-immune serum enhanced macrophage phagocytosis of sporozoites in vitro (75). Macrophages taken from both normal mice and mice immune to *E. falciformis* accumulated sporozoites in culture, but only immune macrophages became activated and digested the sporozoites in a complement-dependent mechanism (8). Macrophages produced a significant amount of TNF in vitro following stimulation with sporozoites and merozoites (118). Thus, it is possible that macrophages modulate the host response to *Eimeria* spp. through production of these mediators.

Mucosal mast cells were also seen to respond during *E. nieschulzi* infection in rats (83). The release of a soluble serine protease was greater in infected rats than in the controls (37). Additionally, the number of mucosal mast cells per crypt and the concentration of serine protease were greater during a secondary response than during the primary response (37).

As discussed above, NK cells are a population of non-T, non-B, nonmacrophage mononuclear cells that exhibit spontaneous cytotoxicity against a wide variety target cells (33, 81). Mammalian NK cells express the asialo-GM_1 antigen and low levels of CD8 (44, 45). It has been postulated that NK cells play an important role as a primary host defense mechanism against tumors, bacteria, and viruses, as well as in the homeostasis of normal tissues (12). Positive correlations have been noted between NK cell activity and genetically determined disease resistance to murine malaria (25, 101) and coccidiosis (57). NK cell activity is augmented following infection with a number of intracellular parasites, including *Leishmania*, *Trypanosoma*, and *Toxoplasma* spp. (12). As with mammals, the chicken intestinal epithelium also contains cells that can mediate NK cell activity (17). Increased NK cell activity occurs in the early stages of coccidia infection (57), suggesting a role for NK cells in the control of parasite proliferation. The levels of NK cell activity in splenic and intraepithelial lymphocytes are higher in infected than normal animals, coinciding with parasite elimination (57). Thus, these cells may be involved in immunosurveillance against eimerian parasites. Enhancement of intestinal intraepithelial NK cell activity following *Eimeria* infection is observed during the post-patent period. This increase in NK activity is accompanied by an increase in the number of IEL expressing the CD8 (60) and asialo-GM_1 (57) antigens. Following the infection of chickens with *Eimeria* spp., there is an increase in the number of asialo-GM_1-bearing cells and cells with NK markers, suggesting that IEL NK cells may be involved in defense against invasion of the gut mucosa by coccidia (57). Further characterization of chicken NK cells will increase our understanding of their roles in mucosal and systemic immunosurveillance against infectious agents.

CONCLUSIONS

Intestinal immune responses to microbial pathogens that lead to protective immunity involve the complex interplay of soluble factors, leukocytes, epithelial cells, endothelial cells, and other physiological factors of the GALT. The research on immunity to coccidiosis demonstrates that immune responses to these parasites are extremely complex. Different effector mechanisms may be involved depending on the stage of parasite development and on whether a primary or secondary response is occurring. It will undoubtedly be some time before a detailed description of immune mechanisms involved in protection against coccidiosis becomes clear. It is likely that these complex interactions have contributed to the difficulties in developing an effective vaccine against *Eimeria* spp. However, recent technical advances in molecular and cellular immunology have facilitated our understanding of the ontogeny, structure, and function of the GALT and led to investigations of the roles of intestinal lymphocyte subpopulations in coccidian disease processes. Research into the avian common mucosal immune system increases in importance as the development of vaccines against intestinal infections such as coccidiosis, colibacillosis, salmonellosis, and intestinal viruses becomes an industry priority. The advent of new molecular techniques to manipulate the genomes of various pathogens and an enhanced understanding of interaction of the GALT with peripheral lymphoid organs will soon enable new approaches to vaccination against enteric pathogens.

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