



Experimental *Eimeria bovis* infection in calves : cellular changes in peripheral blood and lymphoid tissues

by Alwi Muhammad Shatry

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Veterinary Science

Montana State University

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Abstract:

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Elevated sporozoite specific serum isotype levels, in particular IgM and IgG1, were consistent with increased frequencies of PBL's bearing these isotypes, suggesting that the increases may be related to higher frequencies of antigen-specific cell-surface isotypes. Analysis of sporozoite specific antibody isotype activity in culture supernatant fluid of lymphocytes of different tissue origins identified the MLN as the most active site of specific antibody synthesis. In addition, the differential antigen recognition profiles of serum isotypes suggested the preferential generation of antibody clonotypes, and may, in turn, have implications for the identification of immunodominant antigens.

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This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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ABSTRACT

The frequencies of peripheral blood (PBL) and lymphoid tissue T and B cell subpopulations in normal and Eimeria bovis-challenged calves were determined by flow cytometry and the tissue distribution of these cells studied by immunoperoxidase staining. Increases in cells of the BoT4 phenotype were observed in both circulating and mesenteric lymph node (MLN) cells to account for total T cell increases. IgM and IgG1 cells were increased in PBL but no significant increases were observed for any antibody isotype in the tissues examined. Reduced expression of surface BoT8 in PBL of infected calves paralleled similar changes in the MLN spleen and gut lymphoid tissues. Results of in vitro PBL activation with mitogens indicated differences in responses between BoT4 and BoT8. Expression of the latter was markedly reduced after incubation with Con A, PHA and PMA, the magnitude of decreased expression being higher in cultures from non-infected calves. Together, these results emphasize differential lymphocyte subset alterations in the different lymphoid compartments and suggest that the BoT8 subset surface alterations may constitute a significant part of this subpopulation's response to E. bovis infection.

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CHAPTER 1

LITERATURE REVIEW AND BACKGROUND

Background

Coccidiosis is a disease of various animals, including mammalian and avian hosts such as cattle, sheep, rabbits, turkeys and chickens. Coccidial infections generally result in hemorrhagic enteritis leading to diarrhea, dehydration, anemia, weight loss and in some cases death (1).

The causative agents of bovine coccidiosis, which belong to the genus Eimeria, exhibit strong host specificity (2). Eimeria bovis is regarded as the most frequent cause of bovine coccidiosis (3). In 1972, global economic losses were estimated to exceed \$400 million annually (4). To date, no vaccines or satisfactory prophylactic measures against bovine coccidiosis are available besides chemoprophylaxis.

Eimeria bovis infections are initiated in susceptible hosts by ingestion of sporulated oocysts. Oocysts each contain four sporocysts, each with two sporozoites. In the intestinal tract, the oocysts encounter carbon dioxide, trypsin and bile, causing sporozoite excystation (5). The sporozoites penetrate the intestinal epithelium and endothelial cells of the central lacteals (6). The sporozoites then undergo asexual reproduction (merogony, schizogony) to form first-generation merozoites. Fourteen to

fifteen days following the ingestion of sporulated oocysts, meronts reach maturity. First-generation merozoites then reach the large intestine and cecum where they penetrate glandular epithelial cells and develop to second-generation meronts with merozoites. Adjacent epithelial cells are invaded by the second-generation merozoites which differentiate into male and female gametocytes called micro- and macrogamonts respectively. Microgametes subsequently penetrate adjacent cells harboring macrogamonts (6, 7), where fertilization presumably occurs. Each zygote develops into an oocyst by forming an oocyst wall around itself resulting in destruction of the host cell and oocysts are discharged into the lumina of the cecal and large intestine and excretion in the feces as unsporulated oocysts (8). Oocyst sporulation occurs upon exposure to atmospheric oxygen. Sporulated oocysts are infective to a new susceptible host. Lysis of epithelial cells in the large intestine is caused by oocysts, resulting in hemorrhagic enteritis (9).

Surface Host-Parasite Interactions

A monoclonal antibody specific for a 20 kilodalton (kDa) sporozoite surface protein (p20) inhibits sporozoite penetration of Madin-Darby bovine kidney cells and a monocyte cell line (13). P20 has been shown to be an immunodominant surface antigen (14). These findings indicate a potential role of p20 in sporozoite penetration, and may be involved in

initial surface interactions with monocytes and vascular endothelial cells. The immunodominance of this molecule suggests its high immunogenic potential. The binding of p20 by circulating IgG from immune calves may be indicative of a possible mechanism of the expression of acquired humoral resistance, in blocking early surface interactions between sporozoites and host cells in challenge infections. The binding of parasite-specific IgA and IgG on the surface of sporozoites and merozoites has been demonstrated by immunoelectron microscopy (15). The association of the former isotype with structural damage on the sporozoite surface (16) lends support for sporozoite-targeted, antibody-mediated effector mechanisms resulting from host-parasite surface interactions.

In other protozoal systems, immunoblotting techniques have been adapted to the study of molecules involved in interactions between parasites and mammalian host cells (17). This approach has led to the simultaneous identification of Trypanosoma cruzi and host cell molecules involved in the binding interactions. In Leishmania spp., a major glycoprotein (gp63) on the surface of promastigotes (18) and a lipophosphoglycan (LPG; 19) have been shown to mediate parasite binding to and uptake by macrophages. Both molecules serve as ligands for various macrophage receptors among which the complement receptors CR1 and CR3 appear to be most important (20, 21). Immunization against promastigote LPG and

gp63 peptides confer protection to mice challenged with infective promastigotes (22, 23).

The identification of molecules that participate in parasite attachment to and uptake by host cells has, therefore, led to a better understanding of parasite evasion strategies (24) and novel molecular approaches to parasite vaccine design.

Phenotypic Characteristics of Bovine Peripheral Blood and Tissue Lymphocytes

The phenotypic and functional characteristics of bovine peripheral blood mononuclear cells (PBM) have been extensively studied. Typically, monocytes comprise 5-20% of PBM isolated by density gradient centrifugation. The detection of monocytes is based on staining with cytoplasmic α -naphthyl esterase or monocyte specific antibodies (25). Lymphocytes constitute the bulk of remaining PBM cells. The generation of monoclonal antibodies (MAb) specific for bovine lymphocyte subpopulation determinants, in conjunction with flow cytometry, immunofluorescence and immunohistochemical techniques, have facilitated phenotypic and functional analysis of PBM and tissue lymphocyte subsets.

Variable estimates on the frequency of lymphocyte subpopulations in PBL and tissues of healthy and infected cattle have been obtained. The frequency of T cells has been estimated as ranging between 20 and 70% of PBM (25-29) based

on detection by MAb specific for T cell markers equivalent to the human CD2 (28), CD3 or CD5 (25). T cells, similarly, constitute 60-70% of peripheral lymph nodes (25, 26).

The frequency of IgM⁺ cells appears to be the principal one reported in bovine PBM and peripheral lymphoid tissues, the frequency of which ranges between 4 and 30 % (27, 31). This closely approximates the relative frequency of circulating B cells, as revealed by cells positive for surface immunoglobulin (25, 26, 29). The percentage of B cells expressing other (IgG1 and IgG2) surface isotypes is reported to be "low" (25, 31) but supporting data are not available. The availability of B cell isotype-specific MAb have not thus far been applied to the study of lymphocyte differentiation. Available data indicate considerable variation in the frequency of given lymphocyte subsets within and between the different lymphoid compartments. Dramatic differences in subset distribution have been documented in afferent and efferent lymphatics of sheep, a phenomenon attributed to differential migration patterns among lymphocyte subpopulations (32, 115).

In contrast to PBM and peripheral lymphoid organs, quantitative and immunohistochemical studies on lymphocyte subsets in the bovine intestinal tissues have received less attention. A recent study determined the frequency of B cells, T cells, T helper (BoT4) and T cytotoxic (BoT8) subsets (33). Percentages of the various subpopulations were

determined in intraepithelial, lamina proprial and Peyer's patch lymphoid cell suspensions using flow cytometry (33). The data revealed that the sum of percentages of BoT4 and BoT8 subpopulations were in considerable excess over those obtained for total T cell populations. This would suggest the T cell-specific MAb may not have recognized target molecules on the T cell subsets. Conversely, the subsets may not all be expressing markers recognized by the putative T cell MAb, or the T4 and/or T8 reagents may recognize some non-T cells.

No studies on tissue localization of T cell subsets in gut-associated lymphoid tissue (GALT) of the bovine have been conducted. Earlier studies focused attention on the distribution of B cells bearing the surface immunoglobulins, IgM, IgG1, IgG2 and IgA (34-37). Isotype-specific polyclonal antisera and immunofluorescence (34, 35, 37) were mostly used to determine the distribution of surface Ig isotypes. In one study (36), immunoperoxidase staining was applied but the results of all these studies were equivocal. The predominant surface isotypes reported in young calves were IgA and IgM, localized in the lamina propria and the intercryptal region (34). In calves 4 days to 24 months old, IgG2-bearing cells, which were less frequent than the former two isotypes, exceeded the relative frequency of IgG1⁺ cells. In the same study, many cells reported to exhibit membrane IgG1 and IgG2-staining were excluded from the count in favor of cells with intense cytoplasmic staining, which may reflect terminally

differentiated plasma cells. Further, the possible influence of elevated levels of circulating IgG1 in colostrum-fed calves (38) on the frequency of surface IgG1⁺ cells in lymphoid tissues is not known.

In contrast, IgG1 was reported to be the predominant surface isotype expressed on GALT lymphoid cells in heifers 12 to 30 months old (35, 36), the cells exhibiting similar tissue distribution patterns to those described for IgA and IgM. In view of the crucial importance of ileal Peyer's patch in the generation and export of B cells in sheep (39) and its complete involution with age (40) the effect of age on the frequency of surface isotypes remains to be determined. Additionally, the possibility of nonspecific fluorescence or binding of first- and second-step reagents via the Fc receptor (33) was not addressed. Consequently, the contribution of these phenomena to the frequencies observed in these studies remains unclear.

Much of our present state of knowledge on ruminant B cell development comes from studies on lymphocyte migration in sheep. The investigations have led to consideration of the Peyer's patch as a central lymphoid organ for B cell development in sheep (36). The extracorporeal perfusion of isolated segments of the ileum, and the inclusion of fluorescein isothiocyanate (FITC) in the perfusate facilitates labeling of Peyer's patch lymphocytes. The presence of labeled cells in distant lymphoid organs revealed by

fluorescence microscopy, suggested substantial seeding of peripheral lymphoid organs by cells from the Peyer's patch (39, 40). The high turnover of cells in the Peyer's patch (41), the high death rate of Peyer's patch lymphocytes (42) and the severe B cell deficiency in ileectomised prenatal and neonatal lambs (43) are taken as further evidence for the central role of Peyer's patch in the generation of B cells in ruminants.

Immunity-General

Immune responses to parasitic organisms are fundamentally polyclonal in nature leading to the generation of diverse populations of clonotypes, populations of effector cells and molecules with different consequences for host-parasite interactions. While the aberrant immunological phenomena (immuno-depression, autoimmunity) associated with chronic infections with continuously replicating protozoal agents (Leishmania, Trypanosoma cruzi) do not appear significant in mammalian eimerid infections, at least not in E. bovis (44), host related factors (45), including immune status (46), are important in determining the nature of immune responses and disease pathogenesis.

Better understanding of the immunobiology of host-parasite relationships requires analysis of functional interactions between homogeneous populations of antigen-reactive cells and parasite populations. Non-availability of the

latter for any stage in the life cycle of coccidian parasites constitutes a major constraint in this regard. This problem should be circumvented by using molecular approaches in identifying antigens involved in the relevant interactions and cloning genes encoding them. Functional studies utilizing cloned populations of antigen-sensitive T-lymphocytes, although limited in coccidian infections (47, 48), have yielded valuable information on T cell subset function.

Antibody Responses

Most Eimeria species appear to be immunogenic and capable of inducing varying degrees of resistance to reinfection (10). Immunity to challenge infection is manifested as reduced clinical severity and oocyst production; it is generally species-specific (11). Intraspecies immunological variation in the coccidia has been documented (12) and appears to induce partial cross-protection. A parasite-specific IgG response against first-generation merozoites has been demonstrated using indirect fluorescent antibody (49). The response is first detectable 2 weeks following oral inoculation with 10^6 oocysts, peaks at about 21 days (44, 49) and is sustained for up to 98 days (49). In vitro complement-mediated lysis of Eimeria sporozoites and merozoites occurs in the presence of parasite-specific IgG (50). Similarly, a sporozoite-specific IgG antibody was capable of in vitro sporozoite agglutination,

complement-mediated lysis and passive protection against challenge infection with E. tenella (51).

The protective role of circulating antibody remains unresolved but appears to be of relatively minor importance. Past studies on the protective role of antibodies, largely relying on the passive transfer of serum or globulin fractions in mice and rats gave conflicting results (1). Specific antibody titers could not be correlated with reduced oocyst production in immune L3T4 cell-reconstituted mice infected with E. vermiformis (52). Enrichment for antigen-specific antibody and evaluation of different antibody isotypes have not received adequate attention. Quantitative disparities in specific antibody may partially explain the reported variability of passively transferred immune sera in their protective effects. Failure of antigen-specific circulating antibody to accumulate at the sites of parasite development may further account for the apparent ineffectiveness of the passively transferred sera in conferring protection (53). The increased susceptibility to primary E. vermiformis infection in mouse strains with lowered or defective antibody production is not accompanied by failure to develop immunity to reinfection (54).

The demonstration of antigen-specific secretory IgA in mice (16) and rats (55) and the ability of gut contents from immunized chicks to confer some measure of passive protection in avian coccidiosis (56), lends support for a protective role

for secretory antibody. Further, secretory antibody-related ultrastructural damage and reduced motility of E. falciformis sporozoites was observed after incubation with enterocyte-associated mucus from immunized mice (16).

A sporozoite-specific mouse monoclonal antibody (MAb) which recognizes an immunodominant 20 kDa protein (14) on the surface of E. bovis, inhibited sporozoite penetration of cultured bovine monocytes (13), suggesting a potential role for humoral antibody in modulating surface hostcell-parasite interactions, similar to a mechanism that had been proposed earlier for parasite-specific IgA (11). Similarly, sporozoites of E. tenella treated with a specific MAb failed to infect naive chickens, and ammonium sulphate-precipitated ascites fluid injected intraperitoneally protected recipient chicks against challenge infection (57). Of particular interest is the efficacy of a parenterally administered parasite-specific antibody of an IgG subclass, which might not be expected to reach high concentrations at mucosal sites. Inflammation-related leakage of circulating antibody has been proposed as a possible means of achieving high concentrations at sites of parasite infection (9, 58). Additionally, the tissue distribution of a passively transferred MAb in an unrelated recipient host may be altered.

The marked increase in IgA-containing lymphocytes in the lamina propria but not mesenteric lymph nodes (MLN) of E. falciformis-immune mice suggests increased probability of

contact between specific secretory IgA and parasites at their development sites (59). In adoptive transfer studies (60), MLN cells from immunized mice were superior to spleen cells in their ability to confer protection. It is tempting to speculate on the potential contribution of a higher frequency of secretory IgA precursors in MLN than in spleen cell suspensions. The precursors would, presumably, subsequently lodge in the lamina propria of challenged recipients and terminally differentiate into IgA-secreting plasma cells. In this regard, actively dividing lymphocytes conferred protection against E. vermiformis in mice while resting cells or those treated with the mitotic inhibitor, vinblastine, did not (60). This observation raises the possibility of the presence of IgA precursors among the MLN donor cell population, which, upon encountering antigen in recipient mice, are induced to recirculate and preferentially localize at sites of terminal differentiation in the lamina propria (61). Adoptive transfer studies aimed at the critical evaluation of the protective role for B-cells should, ideally, control for surface isotype and frequency of antigen-specific cells in donor tissues. In addition, the potential contribution of antigen presentation by specific B-cells in the activation of T-cells (62) merits attention in the analysis of humoral protective responses to eimerid infections.

Analysis of E. bovis sporozoite and merozoite antigens (63, 64) revealed subtle differences: 1) between merozoites obtained from infected animals and those obtained in vitro from sporozoite-infected cultured bovine monocytes (ref. 63), 2) in the sporozoite antigen recognition profiles of specific serum IgG (obtained by differing immunizing protocols) in immunoblots, 3) recognition patterns of sera from different calves (14, 65). Differences in serum binding profiles reflect diverse repertoires of antibody clonotype specificities and the identification of immunodominant antigens require screening of larger numbers of immune sera that would be more representative of the genetic pool.

Studies with polyclonal sera and MAbs revealed antigens unique to sporozoites and merozoites. Identification of stage-specific molecules should be valuable in the purification of potentially protective epitopes especially in view of the inhibitory effects of a 20 kDa specific MAb on sporozoite penetration of cultured bovine monocytes (14).

In other coccidia, such as infection with Cryptosporidium spp., as in the case with Eimeria spp, studies on passive transfer of immunity yielded mixed results. Immune bovine serum (65, 66), colostrum and MAbs (66) neutralized the infectivity of C. parvum sporozoites for neonatal mice and partially protected them against infection with C. parvum oocysts. The oral administration of MAbs to mice had no effect on susceptibility to infection but significantly

lowered parasite burdens (66, 68). The MAbs, belonging to IgG3 and IgM isotypes, recognized nonprotein and protein epitopes, respectively (67). Neonatal calves fed hyperimmune bovine colostrum, with high concentrations of parasite-specific IgG, IgM and IgA were partially protected against Cryptosporidium infection (69). While the high concentrations of parasite-specific antibody isotypes may, in part, explain the partial protective effect, the contribution of other biologically active substances in colostrum (i.e. cytokines, complement) had not been determined. Parasitological and clinical cure of cryptosporidiosis has also been reported in immunodeficient patients treated with bovine hyperimmune colostrum (70, 71). In contrast, passive colostrum protection could not be demonstrated in suckling mice (72) or calves (73). Similarly, the administration of bovine colostrum failed to influence the course of cryptosporidiosis in immunocompromised patients (74). The problem of evaluating the protective role of humoral immunity is further compounded by the demonstration of parasite-specific serum IgM and IgG in immunocompromised subjects (75), suggesting that the eliciting of a humoral response may be insufficient in the expression of protective anti-cryptosporidial effects. Increased IgA and IgE responses to infection have also been documented (76), the former being considered important in clearing the infection (77).

The roles of circulating versus secretory antibodies in the immune responses to coccidial infections may be difficult to delineate. While both humoral components appear to possess in vivo and in vitro parasite modulatory activities, concentration of secretory immunoglobulins at the site of infection in the intestinal tract favors a more important role for them. It is conceivable, however, that the lytic, opsonic or cytophilic effects of circulating antibody may have a greater impact on the development of infection in circumstances which permit contact between such antibodies and the extracellular, invasive stages, such as changes in vascular permeability that occur within a short time of parasite challenge (102). Additionally, the ability of other isotypes (IgM) to complex with the secretory component in the bovine (36) suggests a potential protective role of this class of antibody on mucous surfaces.

T-cell Mediated Immunity

Several lines of evidence have led to the general conclusion that cell-mediated immunity may be more important than antibody in immune responses to Eimeria species: the induction of delayed hypersensitivity has been demonstrated using different Eimeria antigens in rabbits (103), chickens (104), and calves (78). Similarly, studies with E. bovis have demonstrated antigen-specific blastogenesis and the protective effects of dialyzable transfer factor (TF) from immune calf

lymph nodes (78). Studies in athymic mice (79, 80) and rats (81, 82) subsequently established the critical role of T-cells in acquired immunity to experimental infections with eimerid parasites. Athymic (nu/nu) rats passed 3 times more E. nieschulzi oocysts than did heterozygous (nu/+) controls. In contrast to mouse strains with B-cell-related defects, T-cell deficient mice are completely susceptible to reinfection (80). Furthermore, differences in responses between susceptible (C57BL/6) and resistant (BALB/c) strains were evident during primary but not subsequent infections (80).

Findings in recombinant inbred strains (BALB/c X C57BL/6), leading to the conclusion that resistance to E. vermiformis was not associated exclusively with the H-2 locus (80), were later confirmed in experiments utilizing congenic strains infected with E. falciformis (83), suggesting a potential role in acquired resistance, of hitherto undefined non H-2 genes.

Effectors of DH in murine eimerid infection belong to the L3T4 (CD4⁺) subset in mice (84). Transfer of DH by spleen cells from E. falciformis-recovered donor mice was abrogated by depletion of CD4⁺ T-cells. Spleen cells from acutely infected mice suppressed the DH mediated by immune cells, suggesting the transient generation of a suppressor subset, probably of the CD4⁺ phenotype, also observed in leishmaniasis (85). Abrogation of protective effects was noted in this (84) and another study (86) in experimental infection with E.

vermiformis following depletion of adoptively transferred CD4⁺ cells. The latter study also demonstrated that lowered resistance to primary infection was greater in in vivo CD4⁺- than in CD8⁺-depleted mice.

Supernatants of Con A- and antigen-activated peripheral blood lymphocytes are capable of inhibiting the development of E. bovis sporozoites in cultured bovine monocytes (87, 88) suggesting the participation of nonspecific and specific mechanisms of intracellular parasite elimination. The production of gamma interferon (IFN_γ) by a T-cell clone (47) suggests the generation by antigen of a functional subset probably equivalent to the T-helper1 (89). Phenotypic identity of the clone was not, however, established. IFN_γ has been proposed as the soluble mediator inducing the inhibitory effects on sporozoite development (90). In vivo treatment of BALB/c mice with an IFN_γ-specific MAb resulted in enhanced E. vermiformis infection, the effects of which waned when the MAb was administered between 4 and 7 days postinfection (91), indicating involvement of IFN_γ in the control of primary infection but not the expression of immunity to reinfection. This observation would predict that failure of athymic nude mice to control primary infection may be related, at least in part, to deficiency of this mediator. Further support for the role of IFN_γ in infection with Eimeria spp. comes from a study (92) in which IFN_γ titers in calves were elevated after primary but not challenge infection with E. bovis. This is

not surprising given the increased severity of infection in mice depleted of CD4⁺ cells, a subset of which synthesizes IFN_γ (89).

Taken together, these observations suggest potential roles for both humoral and cell-mediated mechanisms in immunity to Eimeria species, in a manner that need not be mutually exclusive. The predominance of IgA precursor cells in Peyer's patches and other gut-associated lymphoid tissues (GALT, 102) and the presence of other surface immunoglobulin-bearing lymphocytes may have crucial antigen presenting functions for GALT T-helper cells (62). Activation of a subset of these T cells by the antigen-presenting B cells could lead to their elaborating lymphokines which enhance microbicidal and parasite modulatory capabilities of macrophages. Further, there may be temporal and transient patterns to the responses unique to each lymphocyte subpopulation at the infection site during progression of infection.

In conclusion, the biological complexity of coccidian parasites, the host responses they elicit and the effector mechanisms involved, can not be adequately explained based on our present state of knowledge of host-parasite relationships. The role of factors not directly linked to immune response genes, which may also have a bearing on the disease outcome, and how the products of these genes subsequently interact with the immune regulatory networks leading to protective immune

expression is not known. The basis of age-related immunity, a feature of coccidian and other unrelated parasites, remains largely undefined. Similarly, host immune responses elicited by complex parasite molecules of varying biochemical compositions, each with widely differing immunogenic potential will, in all probability, within the available repertoire of responses, include many that are redundant having little to no direct impact on parasite elimination (93).

Rationale

Differential patterns of tissue distribution among different subsets of lymphocytes is a well recognized phenomenon and may have implications for lymphocyte function (94). B cells of blood, splenic and lymph node origin were found to have different requirements for proliferation and differentiation into Ig-secreting cells (95). Cowan I Staphylococcus aureus-stimulated blood and splenic B cells secreted immunoglobulin in the presence of recombinant IL-2 alone, while drainage lymph node B cells failed to elicit plaque-forming cells in response to the subcutaneous administration of the thymus-independent antigen TNP-Ficoll (96). Antigen-presenting cells and con A-stimulated T cells from the spleen induced the secretion of only IgM whereas identical cell populations from Peyer's patches induced high levels of IgA secretion and intermediate levels of IgM and IgG

(97). This implied diverse differentiation pathways of accessory cell populations of different tissue origin.

Both qualitative and quantitative differences have been described for antigens capable of eliciting systemic and local mucosal response after oral administration (98). Oral immunization required amounts of antigen far in excess of those required for parenteral administration of systemic immunity. Intestinal and systemic responses were elicited by the oral administration of small quantities of Escherichia coli pili but not by identical amounts of bovine serum albumin. Proteins capable of oral immunization possess "lectin or lectin-like" binding activities while proteins that are unable to elicit oral immunization do not (98).

These observations indicate that the functional diversity of lymphocyte populations may have a site or tissue-related basis, further implying site-related phenotypic diversity. Further, the differential distribution of different immunoglobulin isotypes in B cells of normal BALB/c mice and the subsequent quantitative changes in these cells in the lamina propria and mesenteric lymph nodes following E. falciiformis infection (59), reflects potential similarities to the above experimental models. The concentration of IgA⁺ cells at the apical part of the lamina propria could facilitate contact between parasite-specific secretory IgA with parasites during their extracellular and intracellular

phases, thereby inhibiting their penetration and/or development.

Studies focusing on qualitative and quantitative characteristics of lymphoid cells in Peyer's patches and MLNs have not been conducted in experimental bovine coccidiosis. The present study examined the frequencies and distribution of subsets of T and B cells in the circulation, Peyer's patches, MLN and SPL in calves challenged with E. bovis, using flow cytometric and immunohistochemical techniques. Such information may provide insight on the comparative development of local responses versus responses distal to the parasite development site and the extent to which changes in peripheral circulating lymphocytes reflect the pattern of events at the local tissue level. Elucidation of these responses in Peyer's patches and MLNs may partially explain the failure of parenteral immunizations using oocyst, sporozoite or merozoite antigens to confer protection against challenge infection (99, 100), or the partial to no protection afforded by the passive transfer of immune serum (101).

Specific Aims

1. Determine the frequency of T and B cell subsets in the peripheral blood of normal and E. bovis-challenged calves using flow cytometry.

2. Determine the frequency of T cell subsets and B cells bearing surface immunoglobulin isotypes (IgM, IgG1, IgG2 and IgA) in the ileum, MLN, and SPL of E. bovis-challenged calves using flow cytometry.
3. Study the distribution of T and B cell subpopulations in the Peyer's patch and in MLN using immunoperoxidase staining.
4. Compare the levels of sporozoite specific antibody isotypes in serum and PWM-activated culture supernatants and to determine antigen recognition profiles of serum antibody isotypes.
5. Determine the comparative tissue-binding characteristics of sporozoites using an ex vivo binding assay.

CHAPTER 2

THE FREQUENCY OF CIRCULATING T AND B CELL
SUBPOPULATIONS IN CALVES CHALLENGED WITH EIMERIA BOVISIntroduction

Infection with Eimeria spp, a gut-associated coccidian parasite, results in partial to complete protection against homologous challenge (1). Experimental infection with E. bovis, the principal causative agent of bovine coccidiosis, induces humoral (44, 49) and cell-mediated responses (44, 47, 78). Previous studies in this regard have focused on measurements of in vitro and in vivo correlates of these responses, namely, antigen-specific blast transformation (47, 78), delayed type hypersensitivity (78) and serum antibody titers using indirect immunofluorescence (44, 49) or enzyme-linked immunosorbent assay (ELISA, ref. 106). Serum antibody assays have, in addition, primarily relied on the demonstration of parasite-specific total IgG during experimental infection. Antigen-specific responses by other antibody isotypes have not been characterized in bovine coccidiosis.

Earlier studies on the identification of bovine lymphoid cells largely relied on the demonstration of their rosette-forming or lectin-binding properties. For instance, T cells were identified on the basis of their capacity to bind

fluorochrome-labeled peanut agglutinin (PNA; 107). However, in addition to binding T lymphocytes, PNA also binds non-lymphoid cells (25).

Availability of monoclonal antibodies specific for bovine T lymphocyte subsets (5, 25, 29) and immunoglobulin isotypes (108, 109) has facilitated studies on the frequencies of lymphocyte subpopulations in the peripheral blood and tissues of normal cows and should permit studies on lymphocyte differentiation. Studies on alterations in circulating lymphocyte subpopulations in cattle experimentally infected with bluetongue (110), trypanosomiasis (111) and in mastitic cows have been made possible by subset-specific MAb. Investigations aimed at characterizing changes in circulating lymphocyte subsets should provide additional insights into cellular responses to infectious agents and should complement functional studies attempting to elucidate immune mechanisms associated with such infections.

In vitro antigenic and antigen-independent stimulation of human T cells revealed the down regulation of CD3 and CD4 on human T cell clones (112). Similarly, blood forms of Trypanosoma cruzi cocultured with human peripheral blood mononuclear cells led to a marked decrease in the surface expression of CD3, CD4 and CD8 on PHA-activated cells (113). Failure to demonstrate cytotoxic T lymphocyte activity in diabetes-prone Biobreeding rats has been associated, in part, to markedly reduced expression of cell-surface CD8. These

results suggest that the density of accessory molecules on T cell surfaces are subject to antigen-specific and nonspecific modulation with functional and regulatory implications.

This study was undertaken to characterize, sequentially, the frequency of circulating T and B lymphocyte subpopulations in calves receiving multiple inocula of E. bovis oocysts. In addition, the mode fluorescence obtained from a fluorescence activated cell sorter (FACS) was used as a measure of in vivo surface expression (114) of these molecules in E. bovis-challenged calves. The study also examined the in vitro effects of lymphocyte activation on the expression of CD4 and CD8.

Materials and Methods

Experimental Animals

One week-old Holstein bull calves were purchased from the Bozeman Livestock Auction. The calves were confined to calf pens with slatted floors in isolation facilities, Department of Veterinary Molecular Biology, for 3 to 4 weeks prior to parasite inoculation. The holding facilities were thoroughly cleaned and disinfected prior to the arrival of newly purchased calves. The diet comprised primarily of a commercial milk replacer fed twice daily. The milk replacer was withheld and oral electrolytes administered to calves developing scours prior to infection with Eimeria bovis. At

termination of the experiment, the calves were sacrificed by stunning and bleeding and then prepared for the aseptic collection of appropriate tissues.

The strain of E. bovis used in this study was originally isolated by Dr. D. M. Hammond (Utah, Dr. Speer, personal communication) and subsequently maintained by serial passage in outbred Holstein-Freisian calves. Primary infection was established by the oral administration of 4×10^4 sporulated oocysts of E. bovis in 5 ml physiological saline.

To ensure patency of the infection, fecal samples were processed for oocyst collection at 18-22 days after inoculation. Age and sex-matched control calves not receiving the inoculum were confined to the isolation facilities during the entire experimental period.

Eight weeks following the establishment of the primary infection, a challenge inoculum of 10^5 oocysts was administered orally at 14-day intervals on three occasions. Venous blood was collected weekly in tubes containing heparin to a final concentration of 10 IU sodium heparin per ml blood. The anticoagulated blood was processed for flow cytometry.

Soluble Sporozoite Antigen Preparation

Eimeria bovis oocysts were separated from calf feces by sugar flotation, sporulated, and then stored in aqueous 2.5% $K_2Cr_2O_7$ at 4°C until further use. The oocysts were further purified from contaminating debris by repeated washing in

Hank's Balanced Salt Solution (HBSS), pH 7.2 to remove the $K_2Cr_2O_7$, resuspending them twice in sodium hypochlorite (Clorox) for 30-60 min and then harvesting the oocyst-rich supernatant. The Clorox was then removed by several washes in HBSS, after which the oocysts were resuspended in HBSS and broken by grinding in a motor-driven Teflon-coated tissue grinder. The resultant sporocyst suspension was then pelleted and sporozoites excysted by resuspension in RPMI 1640 containing 0.25% w/v trypsin (Gibco Laboratories) and 0.75% w/v sodium taurocholate (Sigma Chemical Co) and incubated in a 37°C water bath for 60-90 minutes (87, 117).

Sporozoites were purified by passage over a nylon wool column, enumerated and pelleted. The pelleted sporozoites were then lysed in 100 μ l sterile distilled water, subjected to 5 cycles of freeze-thawing and resuspended in CRPMI to a concentration equivalent to 2×10^6 sporozoites ml⁻¹. The supernatant was filter sterilized and used as soluble sporozoite antigen.

Antibodies

Monoclonal antibodies (MAb) specific for the bovine equivalents of human CD2 (BoT2, IL-A42), CD4 (BoT4, IL-A12) and CD8 (BoT8, IL-A 51) were the kindly provided by Dr. W. I. Morrison, ILRAD, Nairobi, Kenya. The MAb, used as mouse ascites, belong to the IgG2a (IL-A12, IL-A12) and IgG1

isotypes; they were all used at a final concentration of 1/4000 for flow cytometry (Morrison, personal communication).

Bovine immunoglobulin isotype-specific MAb (108) were purchased from Ultimate Conceptions (Millers Falls, MA). These antibodies are specific for heavy chains of bovine IgM (DAS 6, mouse ascites), IgG₁ (DAS 17, culture supernatant), IgG2 (DAS 2) and IgA (DAS 7, mouse ascites). All the isotype-specific MAb belong to the IgG₁ subclass. The MAb DREG 55, specific for the human homing receptor (116), was the kind gift of Dr. M. Jutila; this was used as an isotype control.

Fluorescence Activated Cell Sorter (FACS) Analysis

Peripheral blood mononuclear (PBM) cells were isolated by density gradient centrifugation described by Julius et al. (115). Briefly, the heparinized blood was mixed with an equal volume of cold calcium and magnesium-free Hanks' Balanced Salt Solution (HBSS, pH 7.2) containing 5mM sodium EDTA (Sigma Chemical Co., St. Louis, Mo). Twenty five ml of the mixture were layered over 15ml Ficoll-Hypaque (Histopaque 1077; Sigma) and spun at 350 X g for 45 min at 4°C. The PBM-rich interphase was removed, pelleted and treated with 0.16M NH₄Cl, 0.17M tris, pH 7.65 to lyse contaminating erythrocytes (rbc lysis buffer). The cells were then washed thrice at 100 X g for 10 min to help remove platelets that separated at the PBM-rich interphase. They were then enumerated, assessed for

viability by staining with trypan blue (Sigma Chemical Co.). Viability as determined by trypan blue exclusion, always exceeded 90%. Cell suspensions were subsequently prepared in cold GKN buffer (8g NaCl, 0.4g KCl, 1.77g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0.69g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 2g glucose, per liter deionised water; pH 7.2) containing 0.1 % sodium azide and, heat-inactivated 2% gamma globulin-free horse serum (GGF-HS; Gibco Laboratories, Grand Island, NY). Aliquots of 10^6 cells were pelleted in 12 X 75mm Falcon tubes (No. 2052, Becton Dickinson Labware, Lincoln Park, NJ). All incubation and washing steps were subsequently carried out on ice in these tubes.

In order to block non-specific and Fc-receptor binding sites (33), the cells were resuspended in 100 μ l GKN containing 5% normal rabbit serum, 5% GGF-HS, 2% goat serum 0.1% sodium azide and incubated for 15 minutes, washed once in 4ml GKN and incubated for a further 30 min with 50 μ l of the appropriate primary step MAb dilution in CGKN. For FACS analysis, the isotype-specific MAb were used at the following dilutions: DAS 6, 1/1000; DAS 17, 1/100; DAS 2, 1/500; DAS 7, 1/500). Phycoerythrin-labeled goat anti-mouse IgG (Fab'₂, 50 μ l, 1/100; TAGO Inc., Burlingame, CA.) was added to the cells and incubated for 30 minutes. After the final wash, the cells were resuspended in 1 ml CGKN. Control tubes received the secondary reagent only or were treated with the MAb DREG 55, a mouse IgG1, specific for the human homing receptor (116) as an antibody isotype control. Untreated cells were used for

generating forward-scatter profiles and selection of the cell population to be analyzed.

Single-parameter flow cytometric analysis was performed using the FACSCAN (Becton Dickinson, Immunocytometry Systems, Mountain View, CA). Data acquisition and analysis was obtained using the Consort 30 software. Data was acquired in list mode on 10,000 events. Histogram profiles were based on relative cell numbers and fluorescence intensity. The cell population examined was gated on a scatter profile and excluded most non-lymphoid and dead cells.

In Vitro PBM Activation

To study the effects of in vitro activation on the cell-surface expression of BoT4 and BoT8, aliquots of 1×10^6 PBM were resuspended in 0.5ml RPMI 1640 medium (Gibco), supplemented with 20mM L-glutamine, 100 U ml⁻¹ penicillin G, 100µg ml⁻¹ streptomycin, 10% fetal bovine serum and 5×10^{-5} M 2-mercaptoethanol (CRPMI). The cells were incubated with or without concanavalin A (Con A, 4µg ml⁻¹ final concentration), phytohemagglutinin (PHA, 1µg ml⁻¹; Calbiochem Corp., La Jolla, CA), 30nM phorbol, 12-myristate, 13-acetate (PMA, Calbiochem), 1µM ionomycin (calcium salt; Calbiochem) or soluble sporozoite antigen. The cultures were incubated in 24-well plates (Corning Glass Works, Corning, NY) at 37°C in 5% CO₂-95% air. Untreated cultures received 0.5 ml CRPMI. After 18 hours, cells were harvested by gentle pipetting,

washed twice with cold CGKN and processed for flow cytometry as described above.

Enzyme Treatment

PBM cell cultures were incubated with or without varying doses of proteolytic enzymes to determine the relative sensitivities of surface BoT4 and BoT8. Suspensions of PBM cells at a final concentration of $1 \times 10^6 \text{ ml}^{-1}$ were incubated with α -chymotrypsin (ICN Nutritional Biochemicals, Cleveland, OH) or neutral protease (Boehringer-Mannheim Biochemicals, Indianapolis, IN) at 37°C for 1 hour. The cells were harvested and processed for FACS analysis as described above.

Results

Frequency of T-Cell Subpopulations

In one experiment in which 2 infected and one healthy calves were monitored sequentially, two weeks following the first challenge inoculum, the frequency of T cells in the gated population had nearly doubled, relative to the non-infected control (Fig. 1). Considerable fluctuation in the frequency of T cells was observed during the course of the study. However, significant differences ($p=.01$) were observed between pooled data obtained from additional calves similarly infected, in a separate study (Table 1). The transient increases appear to reflect responses to infection as they

were detectable within 14 days after the administration of each challenge inoculum. Although the levels eventually dropped to near baseline, the relative increases were sustained through most of the experimental period.

The frequency of circulating BoT4⁺ cells roughly paralleled alterations in T cells, the most dramatic increases occurring at 14, 35 and 42 days after the initial challenge inoculum (Fig. 2). Differences ($p=0.1$) were also observed in the pooled data (Table 1). In contrast, relative increases in the frequency of BoT8 lymphocytes did not parallel those of T cells, except on day 35 (Fig. 3). No differences were observed in the pooled data. These results suggest increases in the frequency of T cells are largely due to both absolute and relative increases in BoT4⁺ cells and not due to decreases in BoT8⁺ cells.

Table 1. The frequency of circulating T cells, BoT4 and BoT8 cells in calves 49 days post-challenge with Eimeria bovis oocysts.

Phenotype	Infected ^a % positive (mean + SD)	Controls ^b % positive
BoT2	48.1 ± 10.0 ^c	28.0 ± 6.0 (30.5 ± 18.4)
BoT4	23.8 ± 5.6 ^d	13.8 ± 4.9 (17.7 ± 12.9)
BoT8	13.1 ± 3.8	9.1 ± 1.4 (13.1 ± 6.9)

^an=5 calves.

^bn=4 calves.

^csignificantly different from controls ($p=0.01$).

^d($p=0.1$). Parentheses: PBL values from 9 healthy calves.

Sequential mode fluorescence values of BoT8 were lower in infected than in non-infected controls for most of the sampling intervals (Fig. 4). Surface expression of BoT8 as indicated by mode fluorescence, showed considerable fluctuation in both sets of calves although control levels at some intervals were 4 to 5-fold higher than in the infected and challenged calves. Similar trends were not observed for BoT2 and BoT4. In 9 calves surveyed, the mean T8 mode fluorescence values were 10 to 20-fold higher than in the infected calves at day 42 (Table 2). Although mode fluorescence values were not obtainable in a separate experiment, histogram profiles revealed lowered surface BoT8 expression in PBL of infected calves (Fig. 4A)

Table 2: Comparative levels of surface expression of BoT8 on circulating and tissue lymphocytes of infected and non-infected calves.

Mode Fluorescence				
	PBL	MLN	Ileum	SPLN
Naive	312 (1039±399*)	889	417	1187
Naive	ND	691	416	242
Infected	51	53	44	49
Infected	53	48	61	43

*Mean ± SD Mode fluorescence values obtained from PBL of 9 healthy calves. (ND= not done)

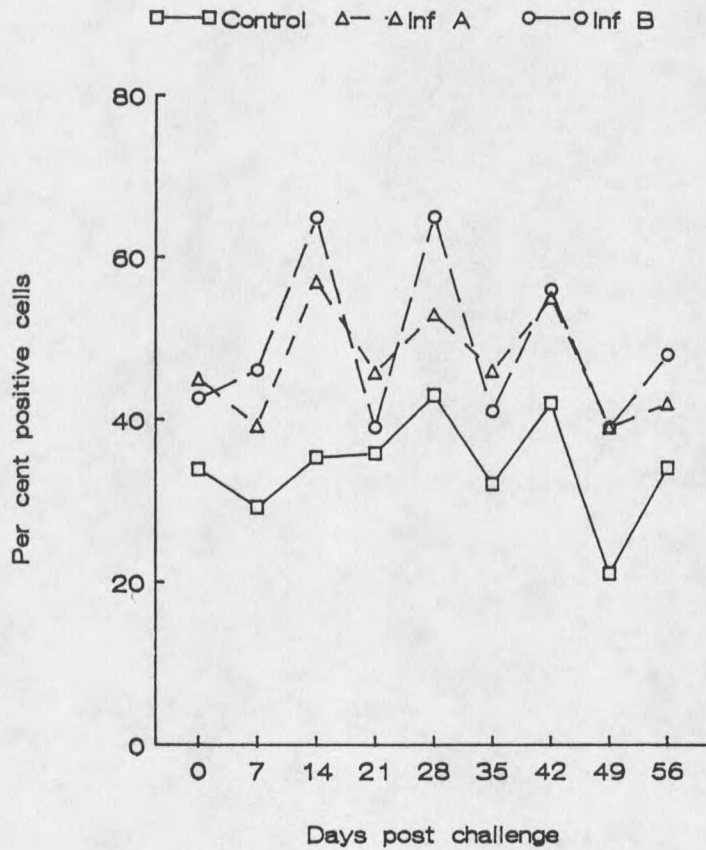


Fig. 1. Sequential alterations in the frequency of circulating T cells in calves challenged with 10^5 *E. bovis* oocysts.

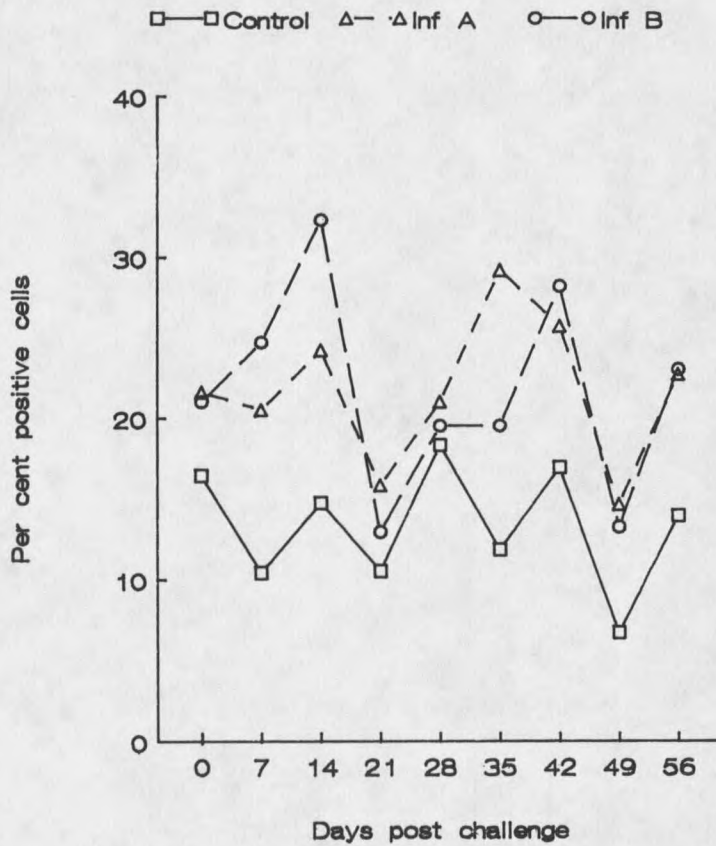


Fig. 2. Sequential alterations in the frequency of circulating Bot4⁺ cells in calves challenged with 10^5 *E. bovis* oocysts.

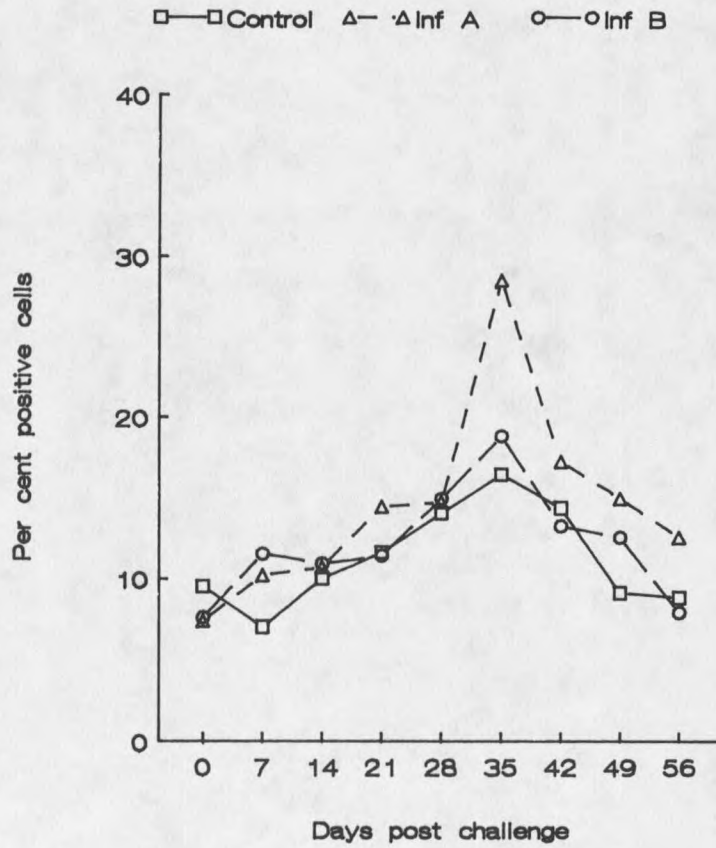


Fig. 3. Sequential changes in the frequency of circulating Bot8⁺ cells in calves challenged with 10^5 E. bovis oocysts.

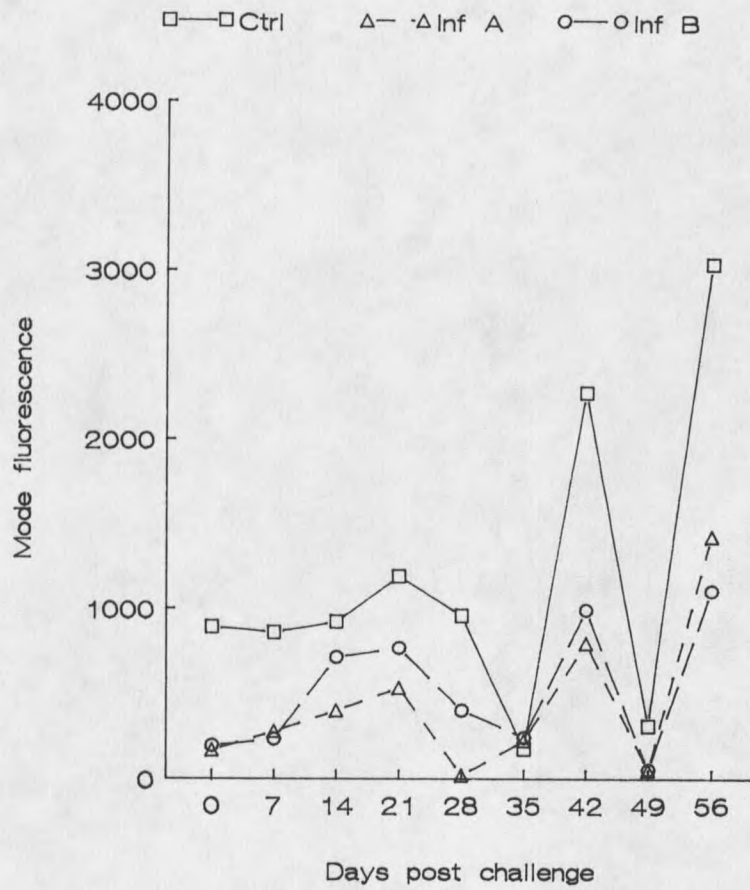


Fig. 4. Sequential cell-surface expression of BoT8 in calves challenged with 10^5 *E. bovis* oocysts.

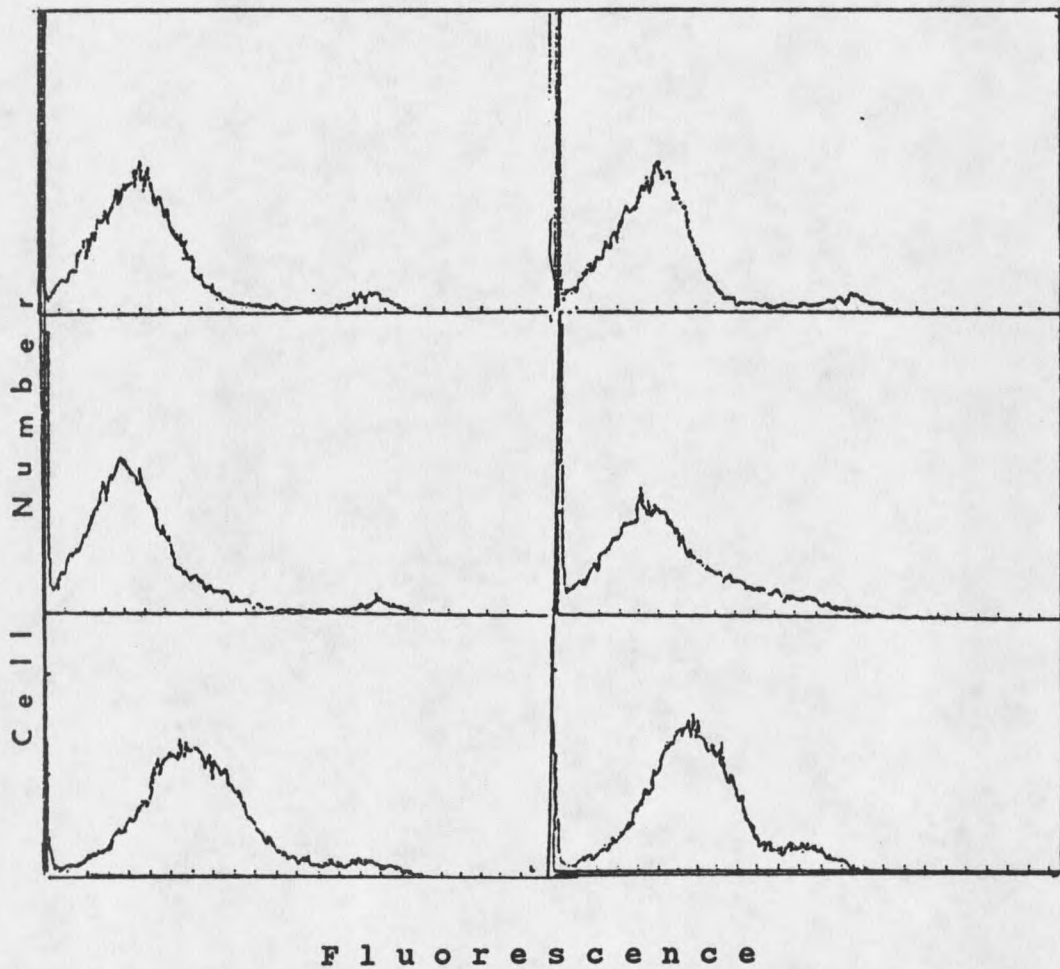


Fig. 4 Continued.

Comparative fluorescence levels of circulating BoT8⁺ cells in 3 control (left histograms) and 3 *E. bovis* - infected calves (right) at 49 days post-challenge.

Cell-Surface Immunoglobulin Isotypes

Circulating lymphocytes positive for DAS-6 (IgM-specific) ranged between 7 to 25% of the gated population in non-infected versus 13-53% in infected calves. Membrane IgM (mIgM) positive cells exhibited a steady increase in infected calves peaking at 35 days following the initial challenge inoculum (Fig. 5). The maximum percentage increase of infected over naive values reached approximately 3-fold at day 49 post challenge.

Lymphocytes positive for the monoclonal DAS-17 (IgG1-specific), likewise exhibited steady increases during the experimental period (fig. 6). The increased frequency of these cells was more marked at days 35 and 49 post-inoculation, being 10-fold higher than the frequency of control cells. The observed levels in both groups of calves were comparable at 0 - 14 days post-inoculation. Cells expressing both the IgM and IgG₁ isotypes were significantly higher ($p=.05$) at day 49 post challenge than either the control group or 9 healthy calves surveyed (Table 3). In contrast to the patterns observed for pan T and surface BoT4, the decline in the frequency of mIgG1 during the intervals between the initial inocula and the challenge inocula was not observed. This suggests the generation of a stable memory mIgG⁺ population which became expanded during subsequent oocyst inoculations. This may further indicate that these

cells are maintained as a stable circulating subpopulation in view of the sustained increases during the intervals between initial and challenge inocula. Alterations in mIgG2⁺ cell frequency were less uniform than those in mIgG1⁺ cells, but increases were more marked at 21 and 35 days post-challenge (Fig. 7). The marked fluctuation in both groups in circulating mIgA⁺ cells suggests transient increases may be random and reflect fluctuations in transiting circulating cells (Fig. 8). Neither of the latter isotypes revealed significant differences from any of the control groups. Histogram profiles for surface T and B cell surface phenotypes are appended (Fig. 17).

Table 3. The frequency of circulating Ig-bearing cells in calves infected and challenged with 10⁵ *E. bovis* oocysts.

Isotype	Infected ^a % positive (mean±SD)	Controls ^b
IgM	23.4 ± 13.8*	7.7 ± 3.6 (10.8 ± 6.0)
IgG1	11.2 ± 11.9*	0.9 ± 0.9 (2.5 ± 2.2)
IgG2	2.6 ± 2.2	0.7 ± 0.3 (0.4 ± 0.3)
IgA	1.4 ± 0.6	0.6 ± 0.2 (0.3 ± 0.1)

^an=5; ^bn=4;

*Significantly different from controls (p=.05). Figures in parentheses represent values obtained from 9 healthy calves.

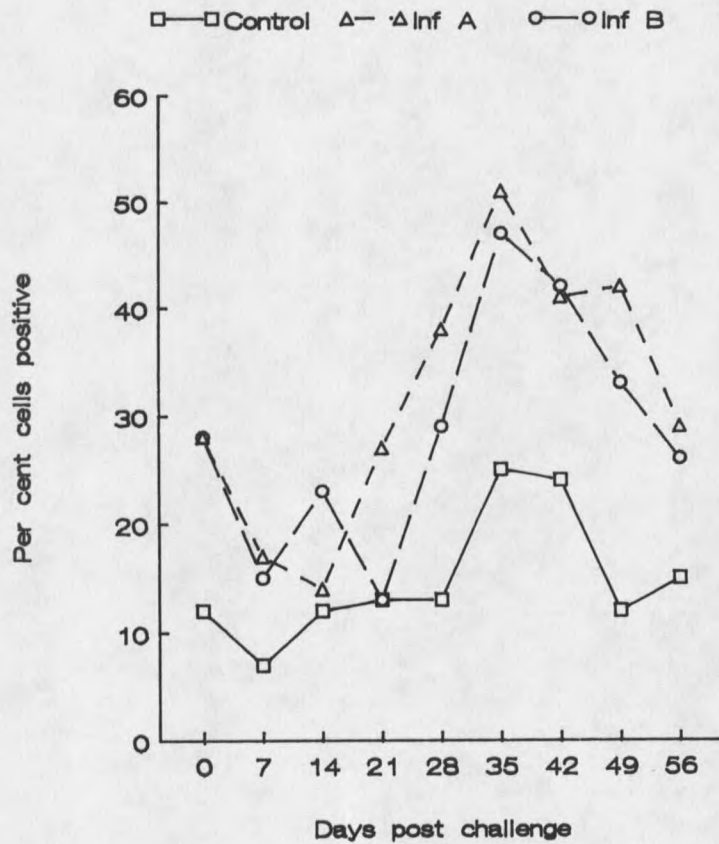


Fig. 5. Sequential alterations in the frequency of circulating mIgM⁺ cells in calves inoculated with 10^5 *E. bovis* oocysts.

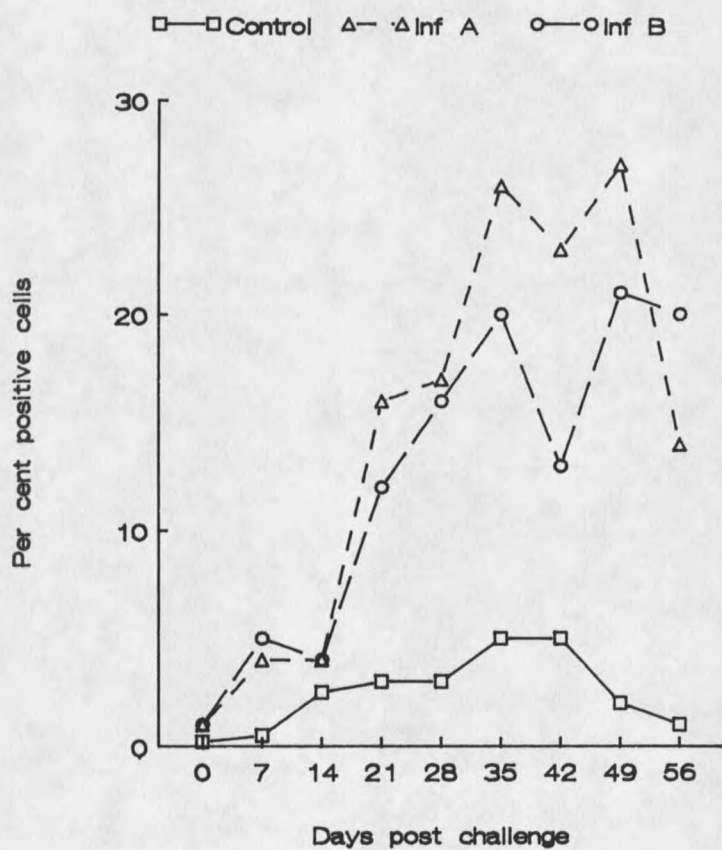


Fig. 6. Sequential alterations in the frequency of circulating mIgG1 cells in calves inoculated with 10^5 *E. bovis* oocysts.

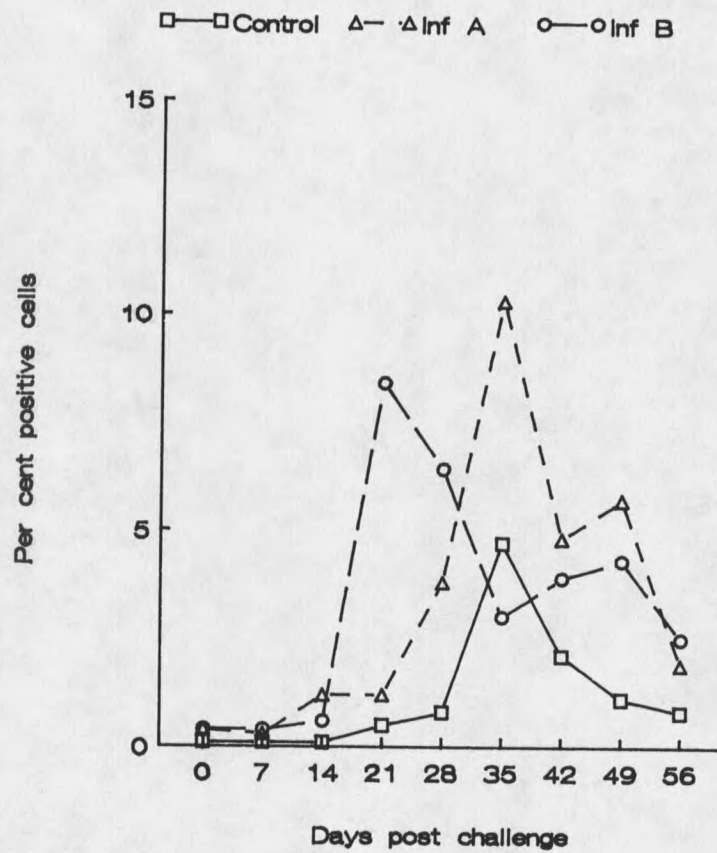


Fig. 7. Sequential changes in the frequency of circulating mIgG2 cells in calves inoculated with 10^5 *E. bovis* oocysts.

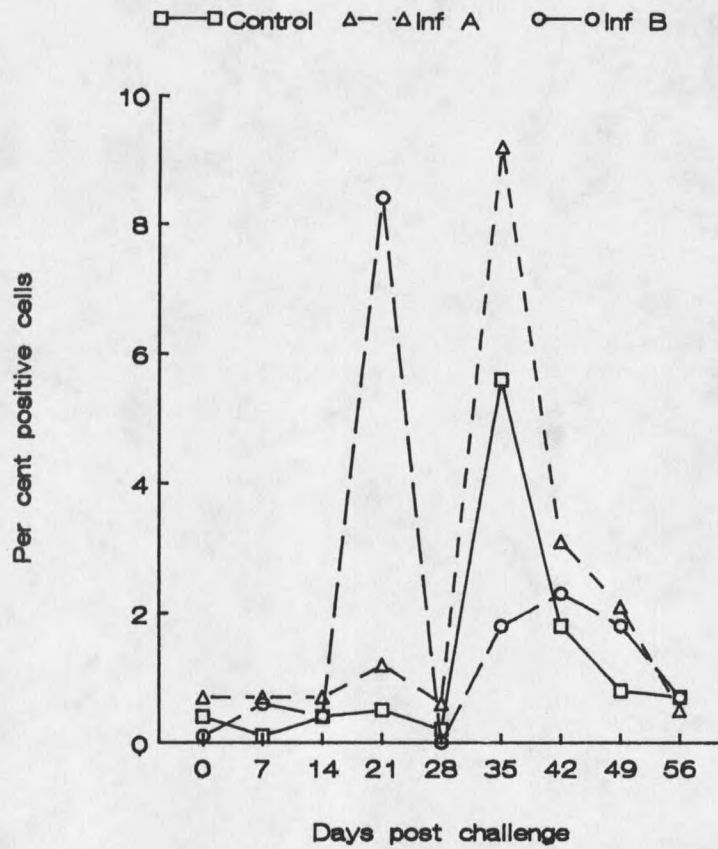


Fig. 8. Sequential alterations in the frequency of circulating mIgA cells in calves inoculated with 10^5 *E. bovis* oocysts.

Expression of BoT4 and BoT8 in Activated Cells

Circulating lymphocytes cultured in the presence of mitogens, the Ca^{++} ionophore ionomycin or soluble sporozoite antigen exhibited differential responses in the surface expression of BoT4 and BoT8. The expression of BoT8 was markedly reduced in cells treated with Con A, PHA and PMA. The reductions, in non-infected cells represented 9 to 17 fold decreases in mode fluorescence values of treated versus untreated cultures (Fig. 9). The decreases in mode fluorescence values of treated cells from infected calves ranged between 3- to 5-fold in one calf (Fig. 10) and 10- to 16-fold in another (Fig. 11). The initial levels of expression of BoT8 in the untreated cells of the former calf may have been a factor in the reduced magnitude of the depressed values. These trends were reproducible in all mitogens tested in two independent experiments, except in one instance where an initial mode fluorescence value of 36 in untreated cells of one infected calf was increased 3-fold in the presence of Con A (data not shown). It should be noted that this low value was comparable to the baseline mode fluorescence values which ranged from 20 to 38 in cells treated with the second step PE-labeled antibody only. In contrast to BoT8, there was variable enhancement in the expression of BoT4 in Con A-treated cells (Figs. 12-14). The mitogens PHA and PMA caused reduced expression in both groups of calves with greater fold

reductions in infected over non-infected mode fluorescence values.

Sporozoite antigen-dependent activation resulted in no alterations in the surface expression of either molecule in cells from a non-infected calf (Fig. 15 A,B). In infected calves, antigen-induced modulation of surface BoT8 resulted in a two-fold increase in the calf with baseline mode fluorescence values but not in the other calf (Fig. 15A). In both calves, however, BoT4 was moderately increased in one and two-fold in the other (Fig. 15B). These results may indicate that antigen activation in vivo renders the surface molecules, in particular BoT8, less susceptible to in vitro antigen stimulation. This may further imply that the in vivo antigen-induced alterations of surface expression resulting from repeated antigen challenge may represent stable and specific responses imposed by antigen, since surface expression exhibited greater sensitivity to antigen-independent (i.e. nonspecific) activation.

Sensitivity of cell-surface BoT4 and BoT8 to two proteolytic enzymes in cells from a non-infected calf revealed dose-dependent reductions in mode fluorescence in the former with both α -chymotrypsin and neutral protease (Fig. 16). Reduction in BoT8 expression occurred only at the higher dose of neutral protease (Fig. 16B) but not with the α -chymotrypsin (Fig. 16B). The differential sensitivity of the two surface

molecules to proteases may indicate differences in regulatory mechanisms of surface expression in the two cell phenotypes.

Discussion

Calves repeatedly challenged with E. bovis oocysts exhibit detectable alterations in the frequency of the majority of circulating lymphocyte subpopulations studied. Increases in T cells are more marked within a fourteen-day period following oral challenge than in the immediate post-challenge period. The fluctuations in frequency in the intervening periods may be an indication of several events occurring in response to the infection: antigen-specific T cells may have selectively expanded upon encounter with antigen. This event, during the induction phase, will conceivably be more accentuated at sites of increased concentration, namely the GALT. As antigen-sensitive lymphoblasts are induced to recirculate, their numbers in the peripheral circulation will likely reflect this event. Since sporozoite release from oocysts and their invasion of the gut epithelial lining occurs within the first 24 hours (Speer, personal communication), the changes in T cell frequencies seen in the peripheral circulation may be a reflection of clonally expanded, recirculating, antigen-sensitized lymphocytes.

Alterations in circulating BoT4⁺ cells closely parallel T cell changes, suggesting that absolute increases in the

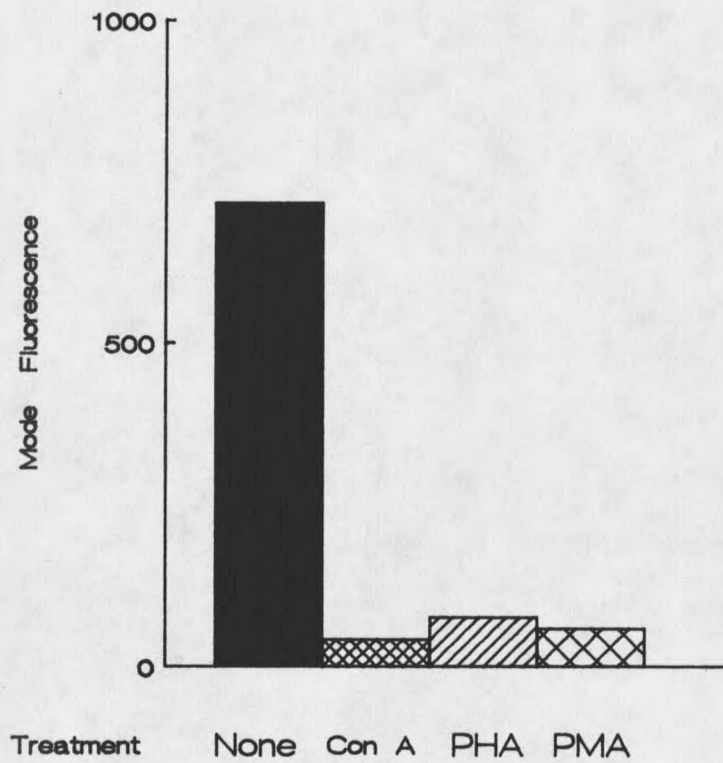


Fig. 9. Cell-surface BoT8 expression on circulating lymphocytes activated in vitro with mitogens, in a non-infected calf.

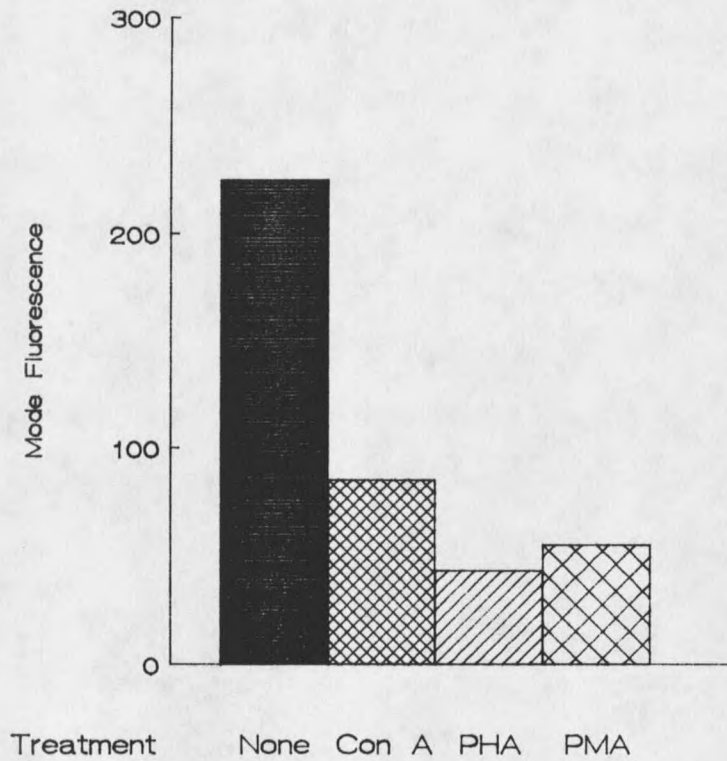


Fig. 10. Cell-surface BoT8 expression on circulating lymphocytes activated in vitro with mitogens in a calf, 42 days after challenge with 10^5 E. bovis oocysts.

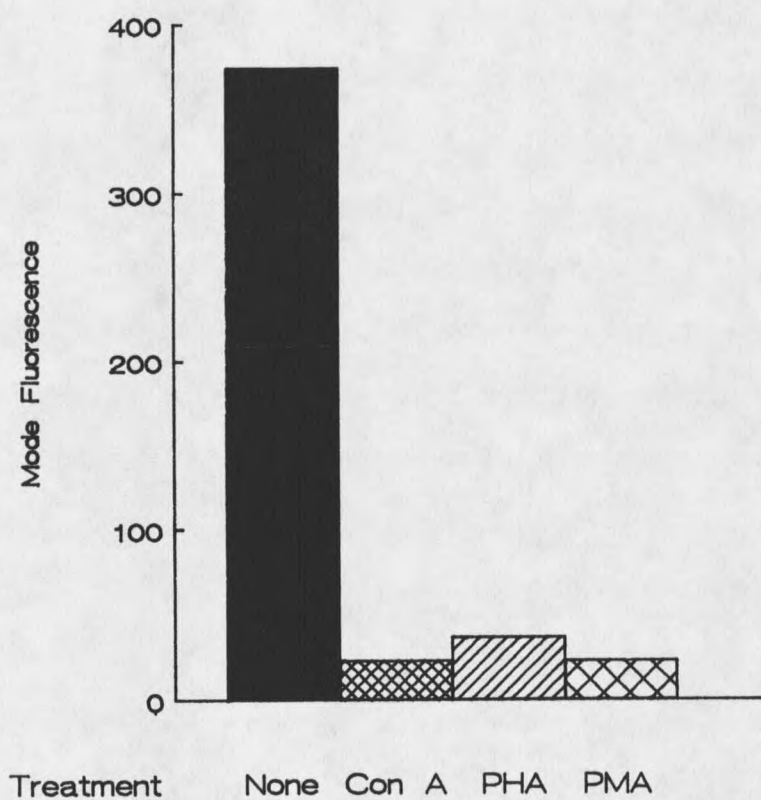


Fig. 11. Cell-surface expression of BoT8 on circulating lymphocytes after in vitro activation with mitogens in a calf 42 days after challenge with E. bovis oocysts.

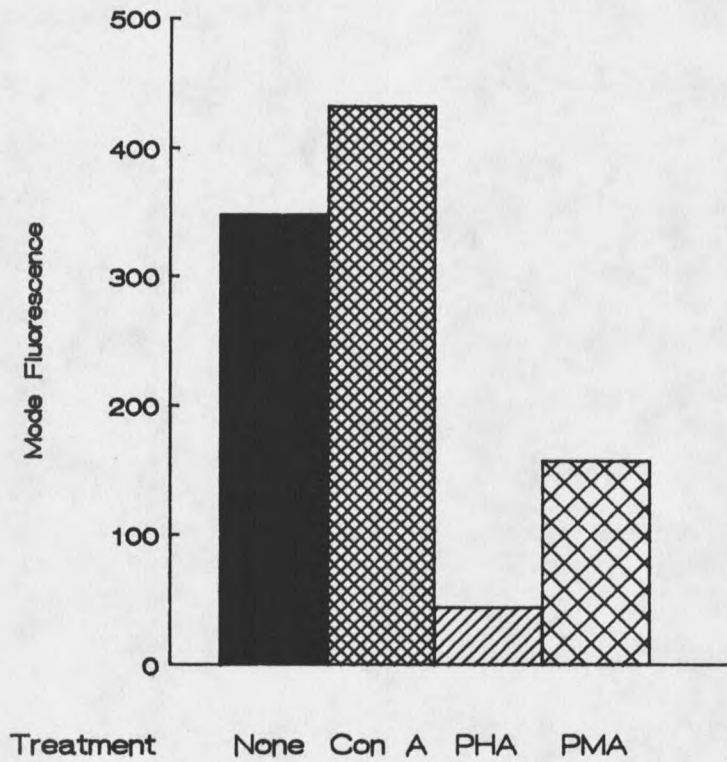


Fig. 12. Cell-surface expression of BoT4 on circulating lymphocytes in a non-infected calf after in vitro activation with mitogens.

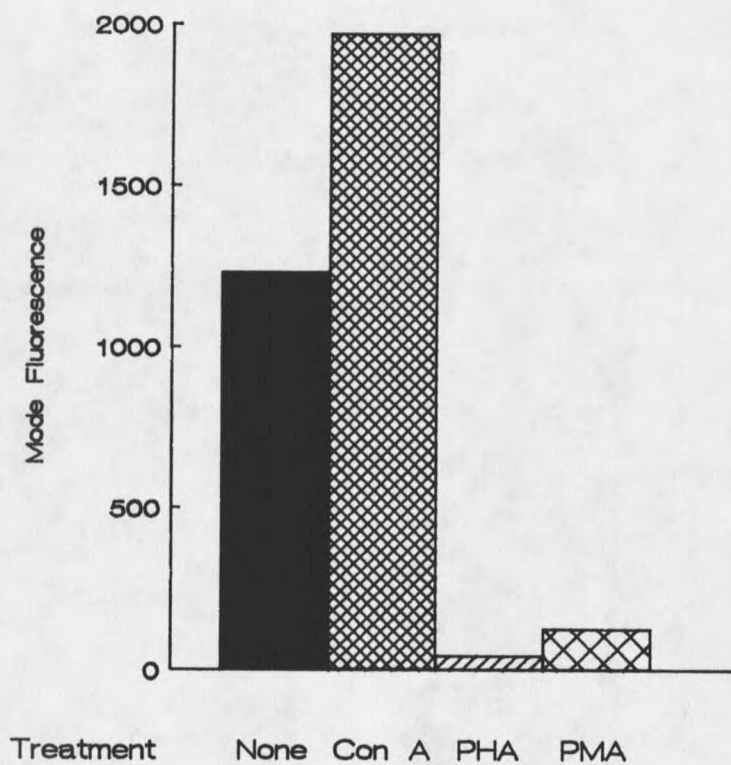


Fig. 13. Cell-surface expression of BoT4 on circulating lymphocytes after in vitro activation with mitogens in a calf 42 days after challenge with E. bovis oocysts.

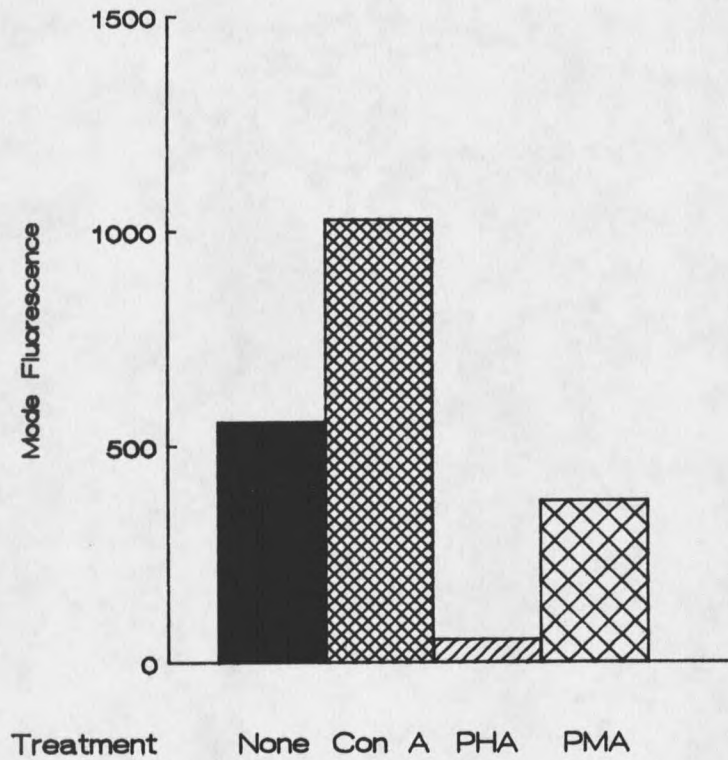


Fig. 14. Cell-surface expression of BoT4 on circulating lymphocytes after activation with mitogens in a calf 42 days after challenge with *E. bovis* oocysts.

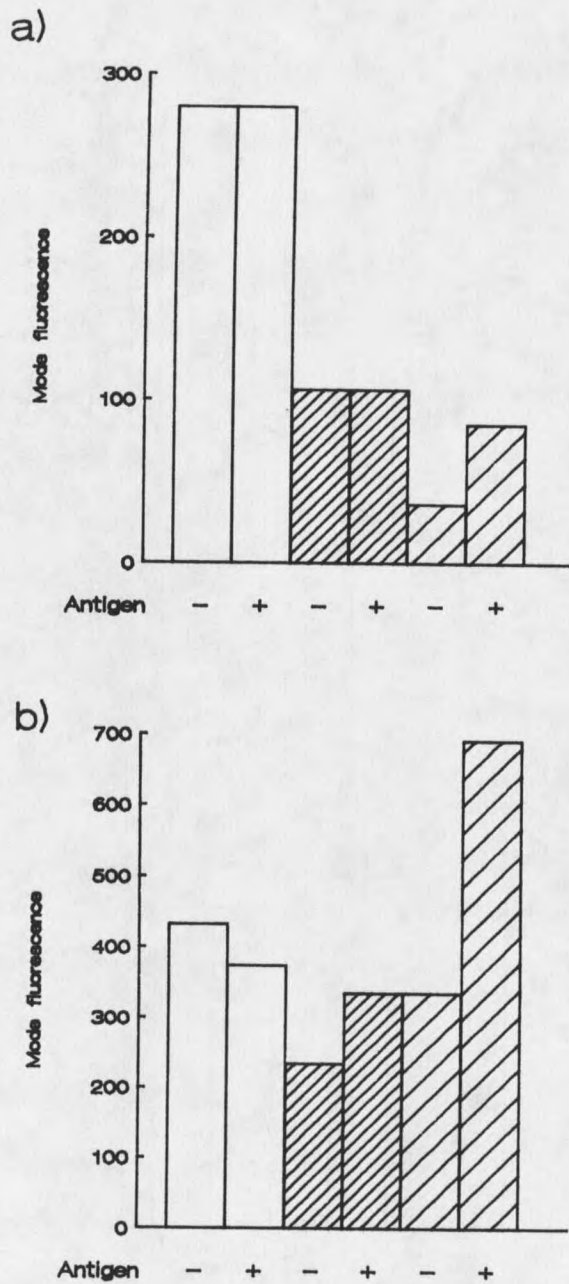


Fig. 15. Cell surface expression of BoT8 (A) and BoT4 (B) in control (open) and infected (hatched) calves. PBM (2×10^5) were incubated for 18 h at 37°C with solubilized sporozoite antigen (2×10^6 sporozoite/well) and processed for cytometry.

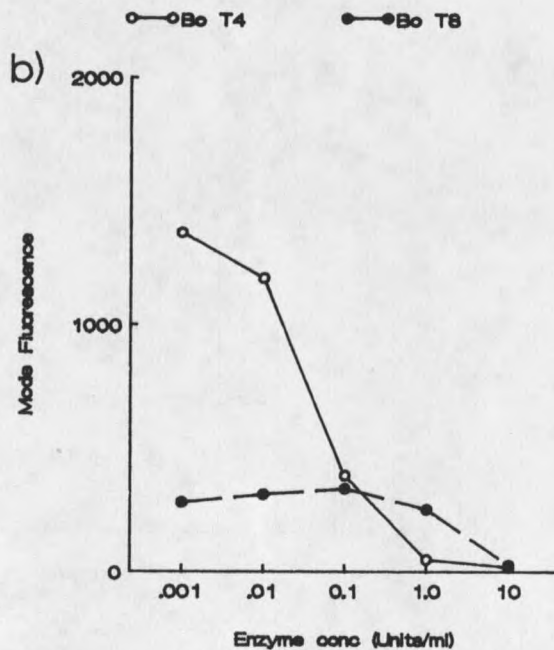
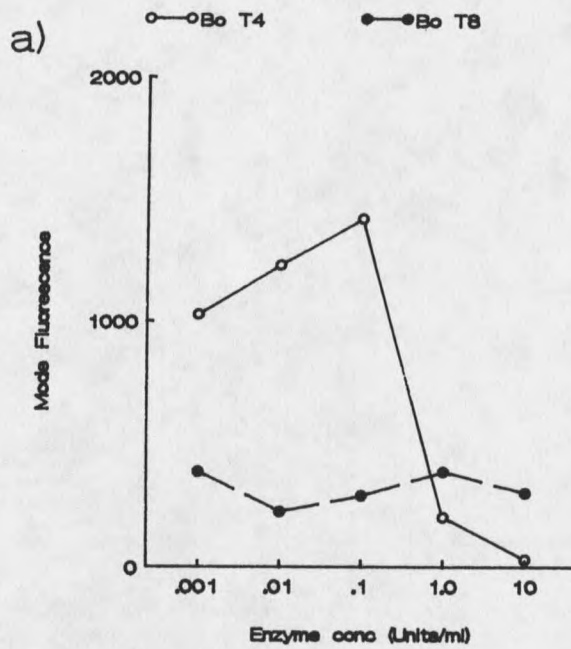


Fig. 16. Effect of chymotrypsin treatment (A) and neutral protease (B) on cell-surface expression of BoT4 and BoT8 on PBL from a normal calf. Cells were incubated with enzyme in serum-free RPMI 1640 for 1 h at 37°C prior to staining for cytometry.

former may be the major factor contributing to the T cell responses. Higher mean T4:T8 ratios in the infected group compared to control ratios for the experimental period concurs with these findings. Marginal changes in the T8⁺ cell populations support the importance of the contribution of T4⁺ cells to the overall changes in peripheral T cells.

The relatively lower levels in the surface expression of BoT8 molecule in infected calves suggests, for this lymphocyte subset, that regulation of surface expression may have more important functional implications than altered frequency in response to the infection. In response to bluetongue virus (110) and trypanosomiasis (111), both absolute increases and decreases, respectively, in frequency have been documented for BoT8⁺ cells. These studies, therefore, suggest the cell frequency of BoT8⁺ is subject to quantitative alteration in response to viral and protozoal infection. The simultaneous increase of BoT4⁺ cell numbers and the decreased expression may reflect the predominance of T-helper-related functions over cytolytic/suppressor functions in parasite-specific, immune effector functions. Of the T-helper-induced mechanisms, the induction of bovine macrophage microbicidal and growth inhibitory activities against intracellular sporozoite by activated T cell supernatants (87, 88), provides a functional illustration in this regard. Further, the importance of interferon- γ infection (91) and the role of L3

T4⁺ cells (86) in murine resistance to experimental E. vermiciformis have been demonstrated.

Steady increases in the frequency IgM- and IgG- bearing cells in infected calves suggests the kinetics of clonal expansion and traffic regulation of antigen-specific peripheral B cells may be different from those affecting cells of the T lineage. Studies on lymphocyte recirculation suggests that differential traffic regulation of lymphocyte subsets, based on their tissue and vascular distribution, occurs in sheep (32, 118). Recirculating specific B cells may continue to expand in the peripheral circulation where they encounter soluble parasite antigen after their initial induction at GALT sites. This, in turn, may be a reflection of, possibly, a relatively long half-life of these antigens, thereby providing a continual source of stimulation for sensitized B cells.

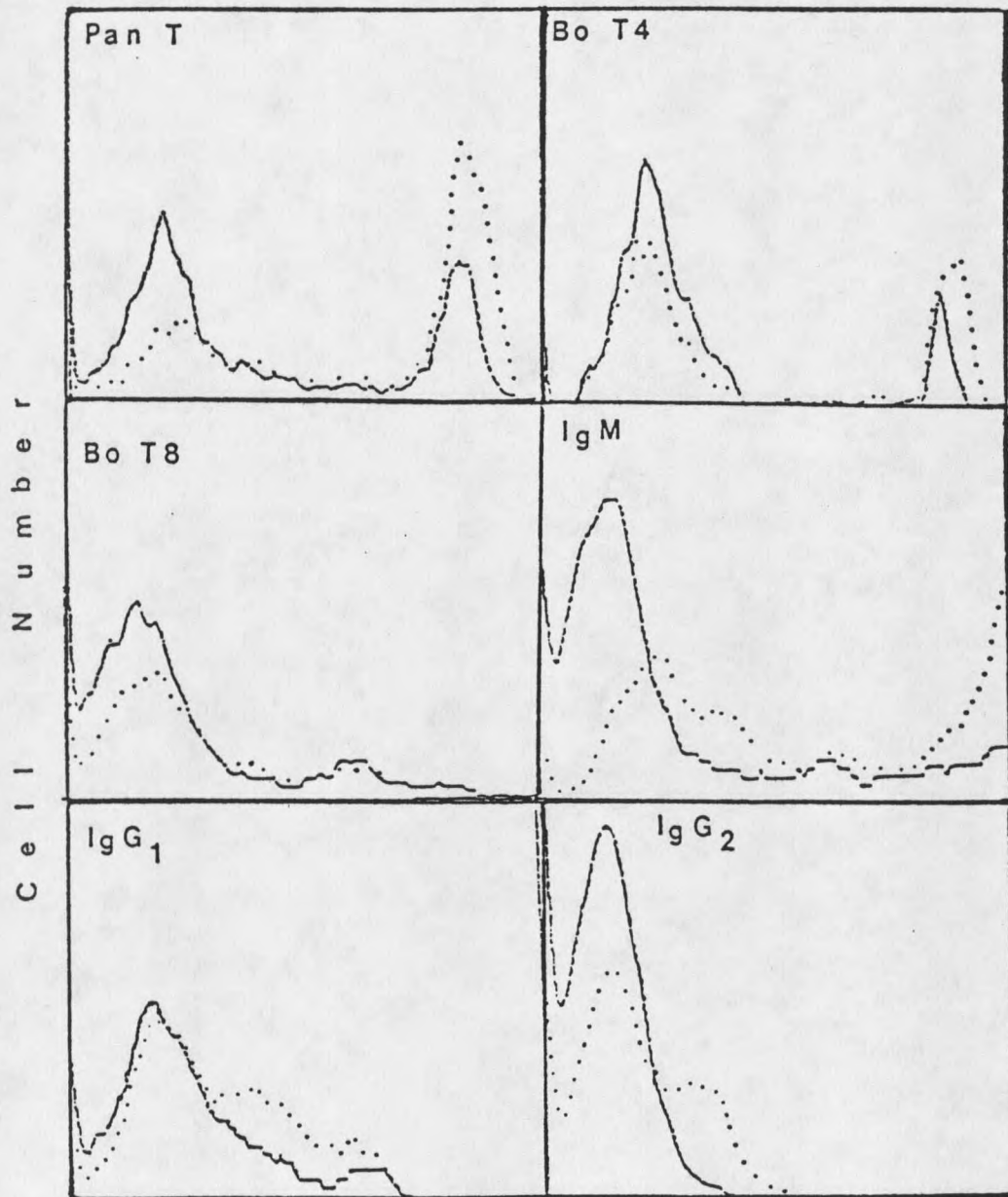
In vitro activation of PBM cells produced variable responses in the surface expression of T4 and T8. Cell-surface expression of T8 was significantly decreased in both groups of calves in cultures treated with Con A, PHA, and PMA. Increased T4 cell-surface expression by Con A, in contrast, suggests that different activation signals may be delivered to the T cell receptor (TcR) on the two subsets by this mitogen. Conversely, this may imply differences in subset TcR, which has been identified as the ligand for Con A (119). PMA caused the down-regulation of T4 but not T8 in mouse

lymphocytes (120). In longer term cultures, however, the expression of surface T8 molecules was also decreased (121). The differences observed in T8 and T4 responses to Con A suggest that the cell-surface expression levels of the two molecules may be regulated by distinct intracellular mechanisms. Treatment with antigen resulted in increased surface T4 expression resembling those induced by Con A, suggesting that mitogenic activation via the TcR by Con A may mimic the effects of antigen-specific activation on surface T4⁺ but not T8⁺ lymphocytes.

Although lymphocyte activation causes receptor phosphorylation and internalization and could partially explain the down-regulatory effects, the modulation of receptors on activated cells by surface proteases could be an additional mechanism responsible for this phenomenon. PMA- and chymotrypsin-induced down-regulation of MEL-14 antigen on neutrophils suggested the potential regulatory role of surface proteases on this cell-surface receptor (122). In this regard, the differential effects of chymotrypsin and neutral protease on surface BoT4 and BoT8 may, in addition to structural differences, reflect differential susceptibility to surface regulatory events.

In summary, increases in the frequency of T cells in calves was largely due to altered BoT4 cells. There were also increases in the frequency of B cells bearing IgM and IgG1 isotypes. Alterations in BoT8 in vivo were principally

qualitative, as reflected in the decreased surface expression of this molecule. Activation with Con A, PHA and PMA mimicked this effect on T8 in vitro. The patterns of altered frequencies of T and B cells suggest differences in dynamics of responses of the two subsets to repeated exposure to E. bovis could be an additional mechanism responsible for this phenomenon. PMA- and chymotrypsin-induced down-regulation of MEL-14 antigen on neutrophils suggested the potential regulatory role of surface proteases on this cell-surface receptor (122). In this regard, the differential effects of chymotrypsin and neutral protease on surface BoT4 and BoT8 may, in addition to structural differences, reflect differential susceptibility to different surface regulatory events.



Fluorescence

Fig. 17. Histogram profile overlays for the frequency of peripheral blood T and B cell surface markers in normal (solid line) and infected calves (dashed).

CHAPTER 3

THE FREQUENCY AND DISTRIBUTION OF
B AND T LYMPHOCYTE SUBPOPULATIONS IN
LYMPHOID TISSUES OF EIMERIA BOVIS-CHALLENGED CALVESIntroduction

Studies on the relative frequencies of B and T cell subpopulations in GALT and other lymphoid tissues in response to experimental infection with E. bovis should provide useful information on parasite-induced alterations at the various tissue level relative to the primary infection sites. Tissue localization of subset-specific surface phenotypes should also shed light on likely differentiation events induced by the coccidia. The predominance of IgA-containing cells in the gut and mesenteric lymph nodes in mice experimentally infected with E. falciformis suggests the relative importance of these cells at primary infection or drainage sites and their preferential generation in response to infection.

In non-ruminants, the Peyer's patch has been found to be an enriched source of IgA precursor cells in the gut lamina propria (105, 123). In sheep, however, the Peyer's patch is regarded as a primary lymphoid organ that exports immunoglobulin-bearing lymphocytes to all other peripheral lymphoid tissues (39). This may imply possible differences in the frequencies of IgA precursors in ruminants compared to other mammals.

In "normal" cattle, variable estimates of T and B lymphocyte subpopulation frequencies have been documented in lymphoid tissues. In peripheral lymph nodes, T cells constitute 60-70% of the lymphocytes (25, 26). The BoT4 and BoT8 phenotypes constitute 40 and 25%, respectively, of lymphocyte suspensions in the same tissue (25). Lower (20-24%) frequencies have been reported for B in cells peripheral lymph nodes and the spleen (26, 124, 125).

In bovine GALT, the frequency of T cell subsets and B cells have been determined for intraepithelial (IE), lamina proprial (LP) and Peyer's patch of the ileum by flow cytometry (29, 33) and immunofluorescence (33b). T cells constituted 44% of Peyer's patch lymphocytes compared to 26% and 38% of IE and LP lymphocytes, respectively. Similarly, the frequencies of B cells and the T-helper phenotype in the LP and IE were twice those observed in IEL. These observations suggest preferential accumulation of lymphoid subpopulations in distinct microenvironments of the bovine gut. These findings were not, however, supplemented with immunohistochemical localization. Certain discrepancies in the frequency of T cells and T cell subpopulations are apparent. In all tissue preparations from the different micro-anatomical sites, the sum of frequencies of BoT4 and T8 cells was in considerable excess over those obtained for total T cell populations, suggesting possible cross-contamination of lymphoid cells from the different sites, the target MAb

epitopes present on PBL T cells may be different from those on IE lymphocytes, the absence of pan T markers on cells of the BoT8 phenotype (33) or recognition of non-T cells by the T cell-specific MAb.

To the best of our knowledge, immunohistochemical localization of domestic ruminant GALT T cell subsets has been demonstrated only in sheep (126). The relative micro-anatomical distribution of B and T cells varied according to the origin of the gut tissue. In the jejunum, the majority of T cells localized in the interfollicular areas, while this site in the ileocecal tissue contained mainly B cells. Some T cells were also present in the dome and the corona regions in tissues of both origins. In the calf, the interfollicular region has been identified as a T cell-dependent area based on the accumulation of labeled cells following the infusion of ^3H -thymidine into the thymic arteries (127).

Earlier studies (34-37) on histochemical identification of immunoglobulin (Ig)-bearing GALT cells yielded mixed results. The tissue distribution of B cells expressing surface IgM, IgG1, IgG2 and IgA has been studied in cattle using polyclonal antisera and immunofluorescence (34, 35, 37) or immunoperoxidase staining (36). The predominant surface Ig isotypes reported in young calves were IgA and IgM located in the lamina propria and intercryptal regions (34). The relative frequency of IgG2⁺ cells in 4 day-old calves, although considerably lower than the former two isotypes,

exceeded that of IgG1-bearing cells. In the same study, many cells reported to exhibit membrane IgG1 and IgG2 fluorescence were not enumerated; only cells with intense cytoplasmic staining were included. These populations may reflect the more mature, terminally differentiated plasma cells, or, in the case of IgM-containing cells, they may also reflect a less differentiated cytoplasmic μ -containing pre-B cell. The latter is less likely in view of the location of positively surface staining cells in the lamina propria or intercryptal regions. Further, the influence of elevated levels of circulating IgG1 in colostrum-fed calves (38) on the frequency and distribution of lymphocytes expressing this isotype is not known.

Other studies reported IgG1 as the predominant cell-surface isotype on GALT lymphocytes (35, 36), exhibiting tissue distribution patterns similar to those described for IgA- and IgM-bearing cells. Factors contributing to the differences observed have not been identified, but, in view of the crucial role of ileal Peyer's patch in the generation and export of B cells in another ruminant (ovine) model (39) and its complete involution with age (40), age-related changes in isotype tissue distribution and frequency may partially contribute to this phenomenon. Furthermore, the possibility of nonspecific fluorescence or the binding of first and second step reagents via the Fc receptor (33) were not addressed in these earlier studies. Consequently the

contribution of these phenomena to the tissue distribution of cell-surface immunoglobulin isotypes described in these studies remains undetermined.

The present study was undertaken to determine the frequencies of T and B lymphocyte subpopulations in the terminal ileum, mesenteric lymph node (MLN) and spleen using flow cytometry in calves repeatedly challenged with E. bovis. Immunohistochemical methods were, in addition, used to study the tissue distribution of surface phenotypes of both cell types in GALT and MLN. These two approaches should be complementary in obtaining quantitative estimates on the relative frequencies and the qualitative immuno-histochemical localization of lymphocytes bearing the various B and T cell markers. The use of MAb specific for T cells, their subsets and Ig isotypes should enhance the specificity of the reactions. Information obtained there-from should improve our understanding of cellular composition and events occurring at primary infection, drainage and distant sites in tissues from infected calves and may indicate likely parasite-induced B cell differentiation events.

Materials and Methods

Experimental Animals and Infection

Calves used in the study and the infection regime used are described in Chapter 2. At 63 days after the initial

challenge inoculum, calves were killed with the captive bolt gun and bled. The skin on both flanks was thoroughly cleaned with disinfectant soap and 70% ethanol prior to the removal of ileal, mesenteric lymph node and splenic tissues.

Lymphocyte Isolation from the
Spleen, Mesenteric Lymph Node and Gut

A portion of the spleen was aseptically removed into cold, calcium and magnesium-free HBSS containing 50,000 IU/ml penicillin, 50,000 mcg/ml streptomycin and 125 mcg/ml fungizone (C-HBSS Flow Laboratories, McLean, VA). After the splenic capsule was removed and the tissue gently teased with a thumb tissue forceps onto a petri dish containing cold HBSS, the suspension was transferred to 50 ml centrifuge tubes. Large tissue debris were allowed to sediment by standing the tubes in ice for 5-10 min., the cell-rich supernatant pelleted and the erythrocytes (rbc) lysed with 0.16M NH_4Cl (rbc lysis buffer). After two washes, the cells were resuspended in HBSS containing 5mM EDTA and 10^7 splenocytes/ml were layered on Ficoll-Hypaque (Histopaque 1077, Sigma) in 15 ml centrifuge tubes and spun at 1000X g for 20 min at 4°C to remove dead cells. Viability was determined by trypan blue exclusion. For FACS analysis, 10^6 cells were used per aliquot. Mesenteric lymph node cells were obtained in a similar manner, from the mesenteric lymph nodes draining the terminal ileum.

For intestinal tissue, a segment of the ileum terminating at the ileocecal junction was identified and freed from the mesentery. Removal of the ingesta was accomplished by flushing the gut lumen with PBS several times using a 60 ml syringe fitted with a 14g needle. This was followed by 3 flushes with PBS containing penicillin, streptomycin and fungizone at the above concentrations. A 6 inch segment of the terminal ileum was then removed and placed in a 300ml bottle containing cold C-HBSS. Further cleaning was accomplished by vigorous shaking the tissue, decanting the C-HBSS and replacing it with fresh C-HBSS. In order to remove epithelial cells, the gut segment was incubated in warm HBSS/5mM EDTA in a 37°C water bath and monitored frequently for the presence of epithelial cells in the buffer. The HBSS/EDTA was replaced for further incubation and the solution monitored for the presence of epithelial cells. This process was terminated when epithelial cells were no longer detectable in the buffer. The EDTA was then removed by several washes of cold HBSS and epithelial cell removal generally required 60-90 min. The peritoneum and residual mesenteric fat was then removed from the gut segment which was then incubated in RPMI containing the mucolytic agent dithiothreitol (10mM; DTT, Sigma) for 30 min at room temperature (128) the tissue minced in cold HBSS to release lymphocytes and incubated for 1h at 37°C in RPMI 1640 containing 45 units/ml collagenase, 60 units/ml hyaluronidase (Worthington Biochemical Corp.,

Freehold NJ). The cells were then washed thrice in cold HBSS and large tissue debris removed in a manner identical to the spleen and MLN. The SPL and MLN cells were also incubated in the enzyme solution. Residual epithelial cells were removed by the rapid passage of the cell suspension over a 0.25g nylon wool column equilibrated with cold HBSS. Non-viable cells were removed as described for the SPL above. Few to no epithelial cells were present in the cell suspensions treated in this manner.

Flow Cytometry

Tissue cell suspensions were processed for cytometry as described above (Chapter 2, Materials and Methods). Briefly, 10^6 cells were incubated in GKN containing 5% GGF-HS, 2% goat serum and 0.1% sodium azide in ice for 15 min to block non-specific and Fc-receptor binding sites. After one wash in GKN, first step antibodies were added to the cells in the concentrations indicated for PBL cells, the cells washed and phycoerythrin-labelled goat anti-mouse Ig (Fab'2, TAGO, Inc., Burlingame, CA) added and the cells washed and resuspended in 0.5 ml GKN for cytometry. Control cells were treated with the second step reagent only or with the irrelevant MAb, DREG 55 (ref. 116). Untreated cells were used to generate forward scatter profiles and select the population for analysis.

Immunohistochemistry

Sections from the ileum and MLN were collected in aluminum foil cups containing embedding medium (O.C.T., Tissue-Tek, Elkart, IN), rapidly transferred to a beaker containing 2-Methyl butane (Aldrich Chemical Co., Milwaukee, WIS), and snap frozen in liquid nitrogen. The frozen tissue blocks were stored at -80°C . Cryostat sections ($4\mu\text{m}$ thick) were fixed in acetone for 5 min, air-dried and stored in slide boxes, under moisture-free conditions at -80°C .

Prior to staining, tissue sections were kept at room temperature for one hour. The tissue area was demarcated with a wax pencil to prevent reagent diffusion. All incubation steps were carried out in a humid chamber, and the slides carefully blot-dried around the sections, between incubations. Tris-buffered saline (TBS, 0.15M, pH 7.60 containing .05% equine serum) was used as the diluent for the primary MAb at the following dilutions: ILA-42, 1/400; ILA-12, 1/400, ILA-51, 1/200; DAS 6, 1/600; DAS 17, 1/200; DAS 2, 1/400; DAS 7, 1/400). The same buffer was also used for rinsing the sections between incubations. Immunoperoxidase staining was performed with a staining kit (Histo-probe, Immunohistological Staining Kit, TAGO, Inc., Burlingame, CA) according to the manufacturer's instructions. Briefly, after $100\mu\text{l}$ of primary antibody was placed on the tissue for 10 min at 37°C , the slides were rinsed and incubated with $50\mu\text{l}$ of biotinylated

goat anti-mouse IgG. After a TBS rinse, the sections were similarly incubated with streptavidin peroxidase, rinsed, incubated with substrate (5 min, 37°C) and counterstained with hematoxylin (3 min, room temperature). After rinsing in TBS, the sections were treated with 50 μ l ammonia water, rinsed in distilled water and one drop of mounting fluid added, followed by the application of a glass coverslip. Control sections received the irrelevant MAb DREG-55 (1/100 dilution) or TBS/equine serum only. The slides were examined with a Nikon Labophot light microscope.

Results

The Frequency of T cells in the Spleen, Mesenteric Lymph Node and Ileum

The frequencies of tissue T cells and their subsets are summarized in Table 4. Mesenteric lymph node cell suspensions from E. bovis infected calves had significantly ($p=0.05$) higher percentages of T cells than MLN from non-infected calves. No significant alterations in this population were evident in the ileum or spleen from infected calves. In addition, the frequency of BoT4 cells was higher in MLN of infected than naive calves. This suggests that alterations in cells bearing the BoT2 phenotype are preferentially increased at this site, and that this is due to increased BoT4 cells. Similarly, in both sets of calves, the frequency of T cells in the SPL and MLN is significantly higher ($p=0.05$)

than in the ileum. This finding is an indication of the differential distribution of T cells in diverse lymphoid compartments of normal and infected calves. Furthermore, although the frequency of BoT8 in identical tissues of infected compared to naive calves was not different, compartmental differences were similarly observed, with the highest frequency of this subset occurring in the spleen.

A three-fold increase in the T4/T8 ratio was observed in the MLN of infected compared to naive calves which further indicates that both absolute and relative increases of the BoT4 subset contributed to the T cell increases (Table 4). Similarly, compartmental differences were observed for this subset, the spleen in both calf groups had higher frequencies of BoT8 relative to BoT4 cells, as indicated by lower T4/T8 values. Although a marginal shift in the ratio also occurred in the spleen in infected calves, the infection did not have a similar impact in the MLN and ileum, indicating the likelihood of a more stable quantitative relationship of the two subsets in this lymphoid organ.

A comparison of the expression of BoT8 in infected and naive calf tissues is summarized in Table 2 (see Chapter 2, Results). In all tissues examined, decreases in surface mode fluorescence values in infected calves ranged between 7-fold in ileal cells to 28-fold in the spleen, with intermediate values observed in the MLN. Similar trends were observed for surface BoT8 expression in peripheral blood lymphocytes (Table

2). Because quantitative estimates for the mode fluorescence were unobtainable, histogram profiles were appended to demonstrate this trend in calf tissues (Fig. 18). These findings indicate that the decreases in surface T8 expression in peripheral circulation are a reflection of similar trends in the tissues.

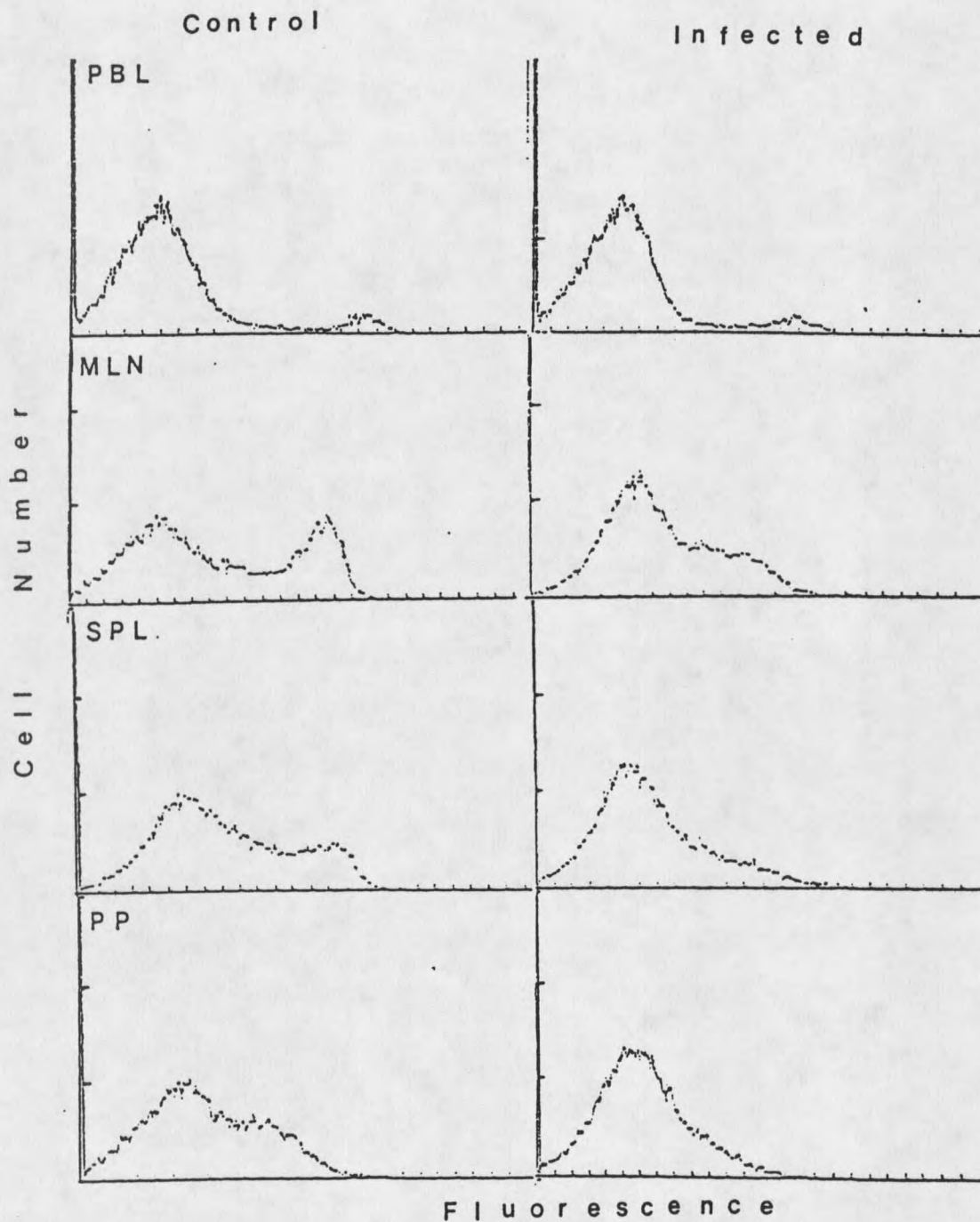


Fig. 18. Relative expression of BoT8 in peripheral blood (PBL), mesenteric lymph node (MLN), spleen and Peyer's patch lymphocytes in control and *E. bovis*-infected calves.

Immunohistochemical Localization of T cells

In the ileum T cells were characterized by random distribution of these cells in the dome areas of the Peyer's patches, and larger, discrete clusters in the interfollicular tissue. The rest of the follicular Peyer's patch contained scanty numbers of BoT2⁺ cells, in some instances giving the impression of being almost devoid of T cells, depending on the level of sectioning (Fig. 19A,B). Considerable numbers of T cells were diffusely distributed throughout the lamina propria and intercryptal regions. The intraepithelial region, especially the glandular crypts were also infiltrated with T cells. Subsets belonging to the BoT4 and BoT8 phenotypes closely paralleled those of BoT2 in their tissue distribution in the ileum (Figs. 20, 21). Intraepithelial localization appeared more prominent with T8 cells (Fig. 21B) which were also less concentrated in the dome and interfollicular areas than T4 cells. These findings suggest the existence of discrete T cell-dependent interfollicular and dome areas in the ileum, similar to sheep (126) and the mouse (127). About 40% of sheep ileal Peyer's patch dome cells were positive for surface IgM (126). Visually discernible differences in the localization of T cells were not apparent between tissues from infected and non-infected calves.

In the MLN, distribution of T cell populations conformed to those described elsewhere (25) namely, the vast majority

of these cells being localized in the cortical and paracortical areas (not shown). Fewer cells expressing T cell and subset markers were located in the deeper cortical or medullary areas. The concentration of cells in these areas varied between lymph nodes of different calves and between different cortical regions of the same node, but in general, the distribution patterns remained largely confined to the cortex and paracortex.

Table 4. The frequency of T cell populations in the ileum, mesenteric lymph node and spleen of E. bovis-infected and naive calves.

Percent positive cells (Mean±SD)						
Ileum		MLN		SPL		
Infection	+	-	+	-	+	-
Bo T2	9.9±2.1	10.7±1.5	44.1±9.4*	23.6±13.5	46.0±7.6	40.8±8.0
Bo T4	9.4±4.9	9.1±1.4	25.3±7.7*	9.9± 3.6	10.6±3.2	8.4±2.0
Bo T8	6.2±5.1	7.3±4.2	8.7±3.8	13.5±10.3	15.0±5.6	17.6±5.7
T4:T8	3.4±3.4	1.7±0.9	3.2±1.1*	0.9± 0.4	0.9±0.8	0.5±0.2

n=5, infected; 4, controls.

*Significantly different (p=0.05) from control values.

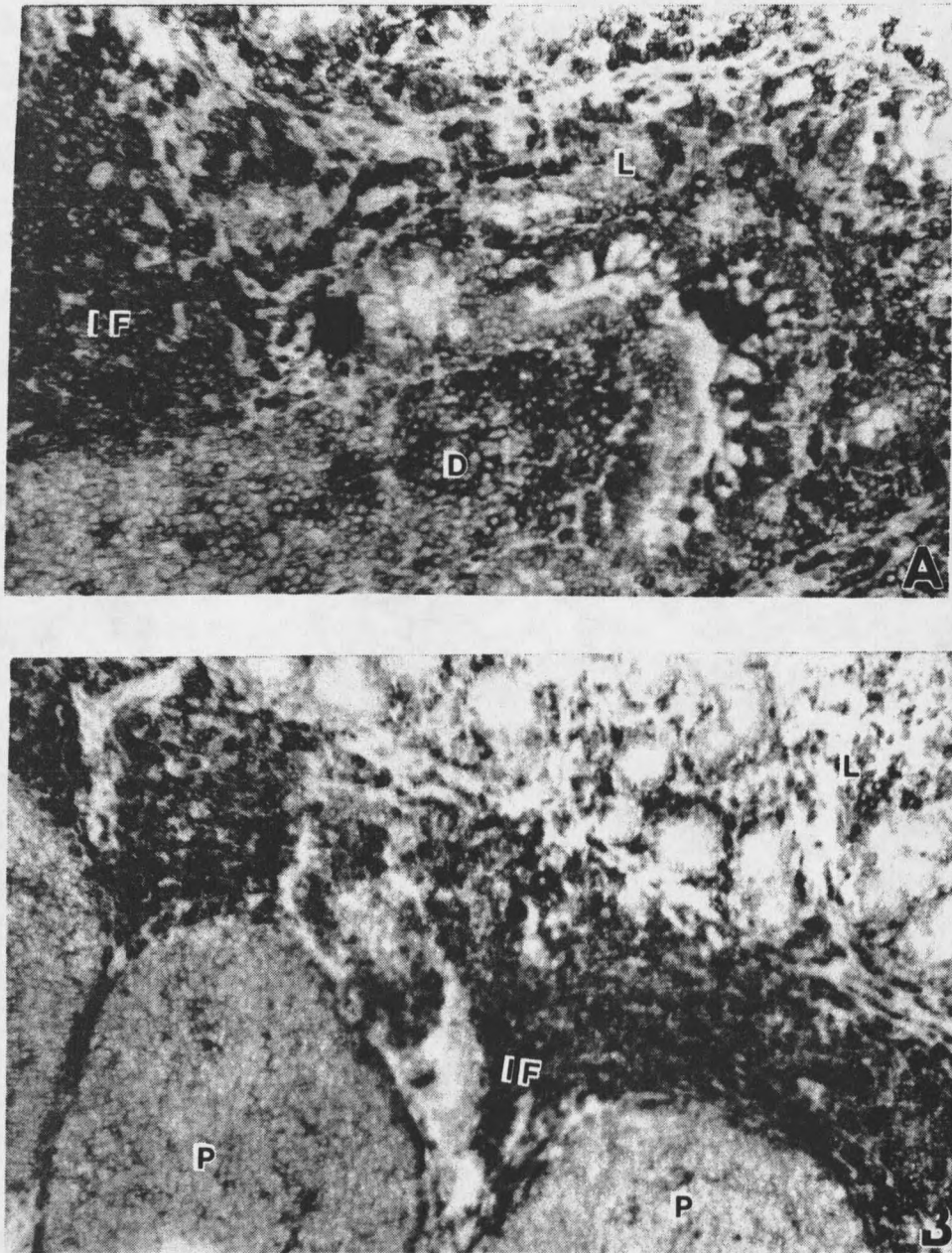


Fig. 19. A. The distribution of Bo T2⁺ cells in the terminal ileum. A) Positive cells are localized in interfollicular (IF) and dome (D) areas of the peyer's patch and intercryptal areas of the lamina propria (L). X160. Fig. 19 B. Note the high density of positive cells in the IF areas and fewer cells in the peyer's patch follicle (P). X100.

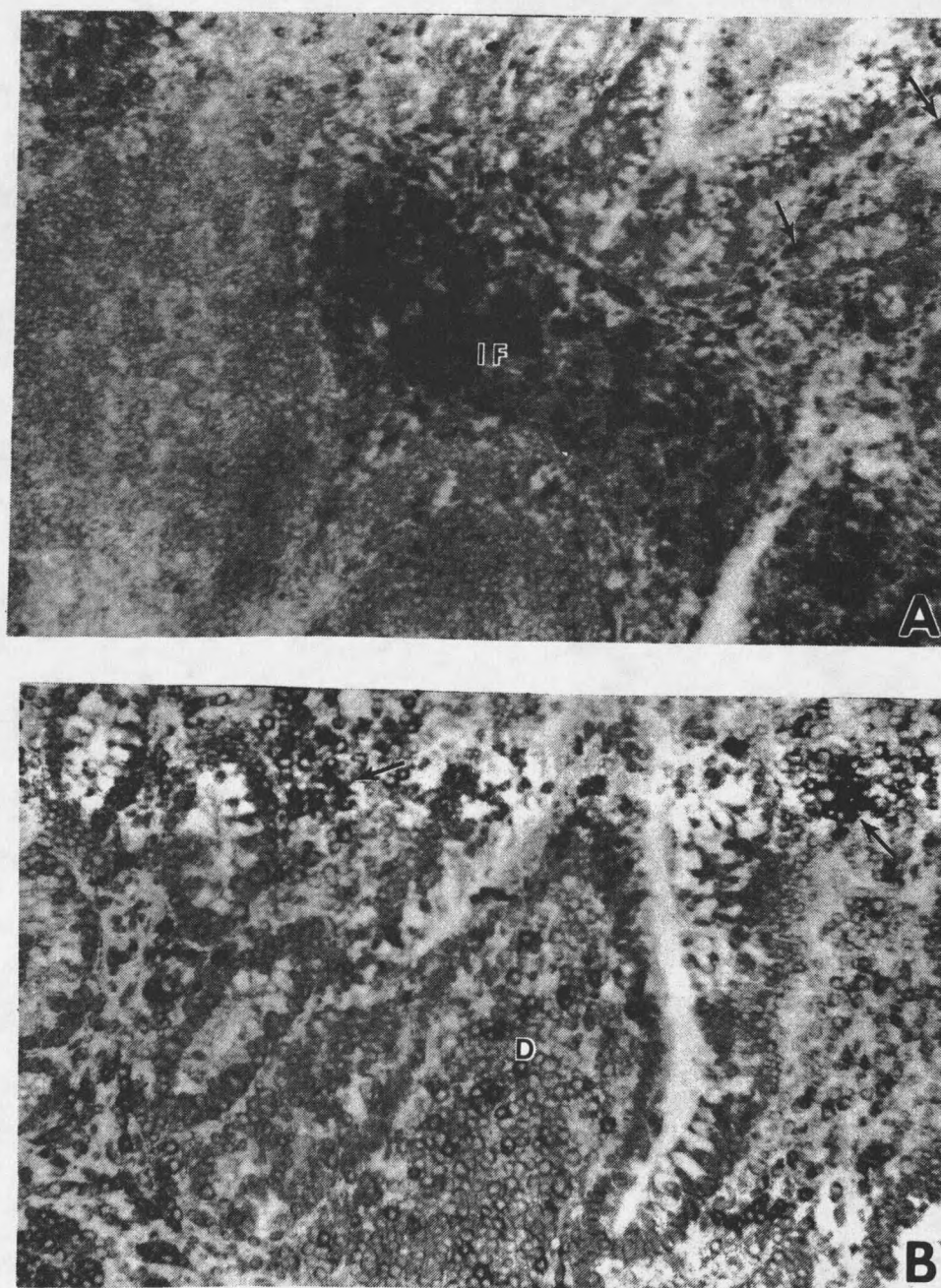


Fig. 20 A. Bo $T4^+$ cells in the terminal ileum are mostly located in the interfollicular (IF) region. Other positive cells are randomly distributed in the lamina propria (arrows). X100. Fig. 20 B. The distribution of Bo $T4^+$ cells in the dome region (D) of the ileal peyer's patch and lamina propria (arrows). X160.

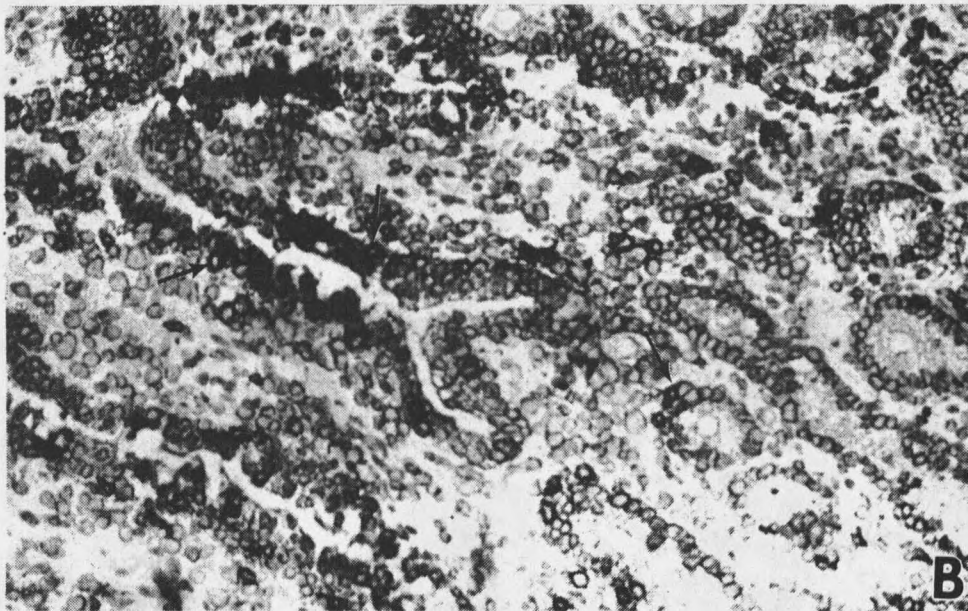
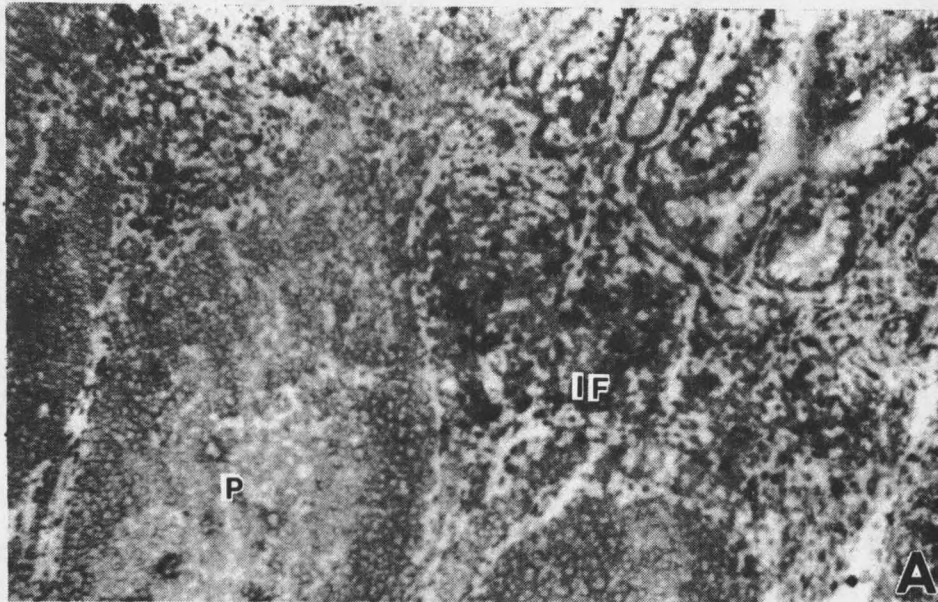


Fig. 21 A. BoT8⁺ cells in interfollicular (IF) areas of the Peyer's patch. Note absence of these cells in the Peyer's patch (P). X100. Fig. 21 B. In the ileal mucosa, positive cells are located in the intraepithelium (arrows), or randomly distributed in the lamina propria. X200.

The Frequency of Ig Isotype-
Bearing Cells in the Ileum, SPL and MLN

The frequencies of B cell subpopulations are summarized in Table 5. Although no significant differences ($p=0.05$) were apparent between identical tissues from infected and naive calves, the frequency of IgM⁺ cells in the ileum was significantly higher than in the spleen and MLN from infected calves. Similarly, the ileum had higher IgG₁- and IgA-positive cells than the spleen and MLN, indicating the differential distribution of numbers of Ig-bearing cells in the different lymphoid tissue compartments. The reasons for the disparity between alterations in circulating IgM⁺- and IgG₁-positive and those in tissues are not clear. A possible explanation may be related to the fact that recirculating cells of GALT origin may not necessarily return to sites where tissues were sampled. Relocation of these cells to other sites in the intestinal tract or other mucosal sites (131) may have a dilution effect on the concentration of cells in the GALT tissues sampled.

Localization of Ig Isotype-Bearing Lymphocytes

In the ileum, surface IgM⁺ cells in the submucosa are located in Peyer's patch domes, in intrafollicular spaces and randomly distributed throughout the lamina propria between glandular crypts and fewer numbers of positive cells could be

observed in the intraepithelial spaces (Fig. 22A-C). In addition, the stain was more intense on the glandular epithelial luminal surfaces. The periphery of Peyer's patch follicles contained the highest numbers of positively staining cells, especially in the quiescent follicles; however, the intensity of staining was considerably decreased compared to positive cells in the submucosal areas. This phenomenon has been described in sheep (126). Similarly, more intensely staining IgM⁺ cells associated with germinal centers were peripheral to this structure.

IgG1-, IgG2- and IgA-bearing cells were similarly located in the dome areas in larger numbers (Figs 23-25) and the former two isotypes more commonly detected in the intraepithelial spaces. Although intraepithelial localization was not observed for IgA⁺ cells, the luminal epithelial surfaces stained most intensely in sections incubated with the MAb DAS 7 (anti IgA) compared to the other isotypes. In Peyer's patch follicles, localization of the IgG- and IgA-bearing cells were localized on the peripheral zones of the germinal centers (Figs. 23B, 25B). The observations described here suggest subtle differences in the tissue localization of Ig-isotype bearing cells in calves, with fewer cells of the IgG and IgA isotypes localized in interfollicular T-dependent areas. As in the case of T cells, there were no discernible differences in distribution patterns between tissue sections from infected or naive calves. Further, germinal center

activity was also evident in some Peyer's patch sections from naive calves, making it difficult to distinguish between E. bovis- or environmental antigen-induced changes.

In the MLN, cells expressing surface Ig isotypes exhibited some differences in distribution. Surface IgM⁺ cells were predominant and localized mainly in the cortical B-dependent areas; they were also detected in primary follicles in cortical or medullary areas (Fig. 26A,B). Cells bearing the IgG isotypes were mostly associated with more mature follicle types or germinal centers in the deep cortex (Fig. 27A,B). The distribution of surface IgA⁺ cells was random and were detectable as scattered solitary cells in cortical or medullary areas (Fig. 27C).

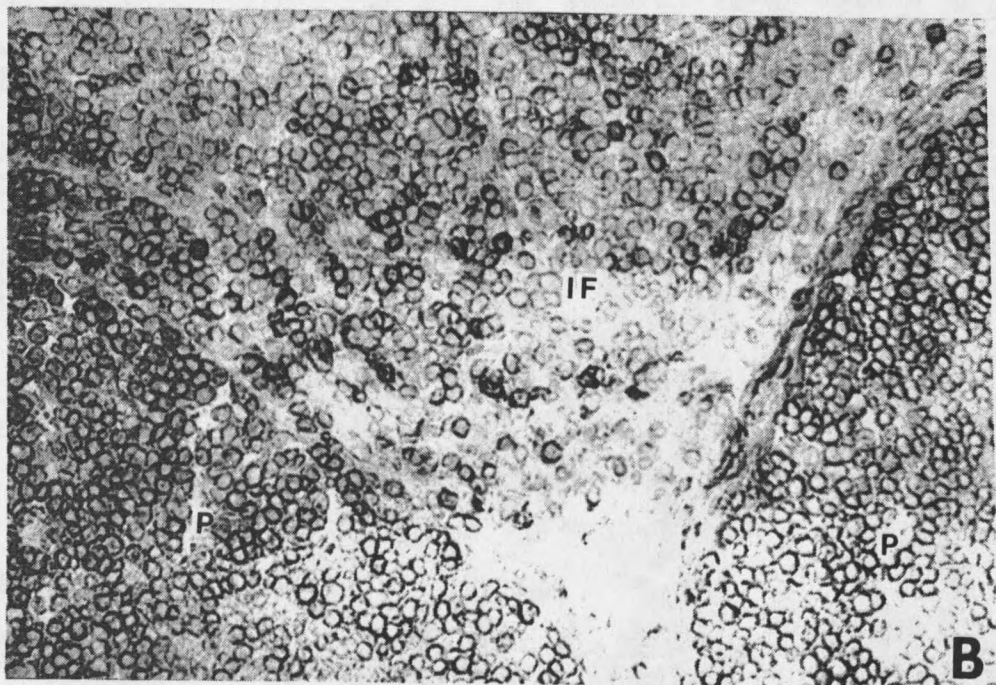
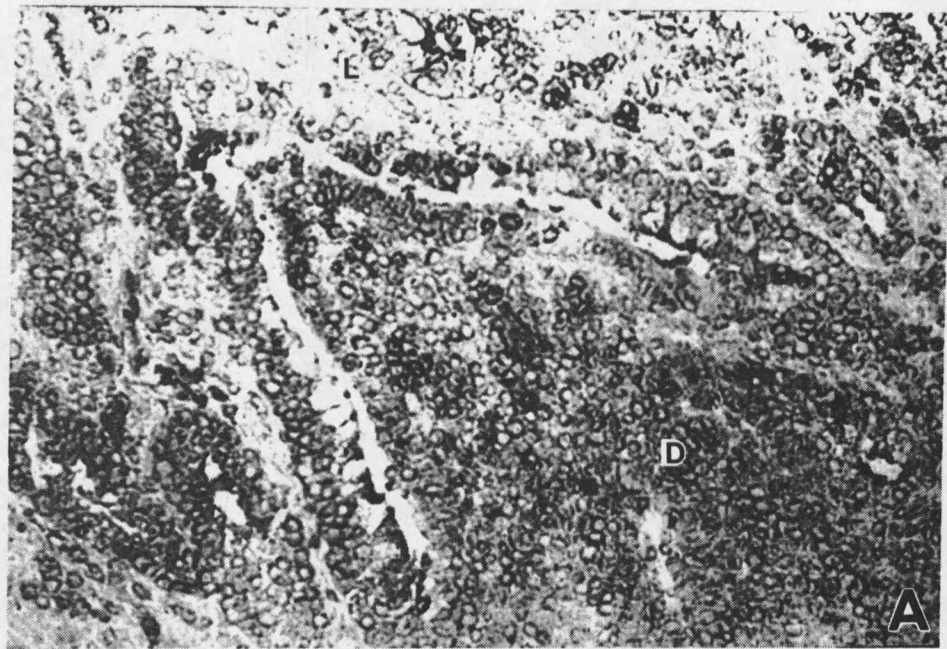


Fig. 22 A. Localization of IgM^+ cells in the dome (D) of ileal Peyer's patch and lamina propria (L). X160. Fig. 22 B. Interfollicular (IF) and Peyer's patch (P) distribution of these cells. Note disparities of staining intensity on positive cells in adjacent Peyer's patch follicles. X200.

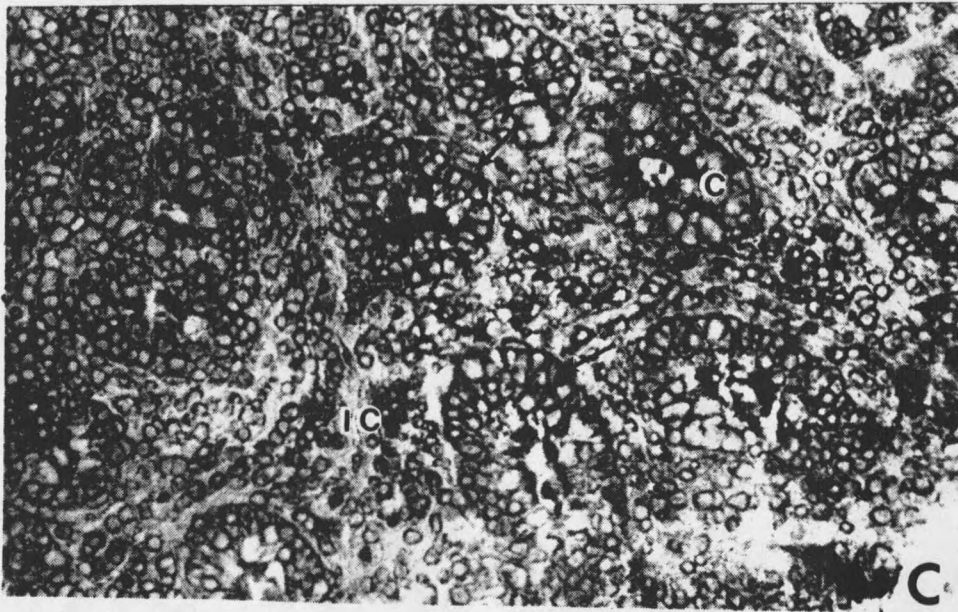


Fig. 22 Continued.

C. The distribution of IgM⁺ cells in the ileal mucosa. Positive cells are randomly distributed in the intercryptal spaces (IC) and some are intraepithelial (arrows). Note intensely stained epithelial borders of the crypts (C). X200.

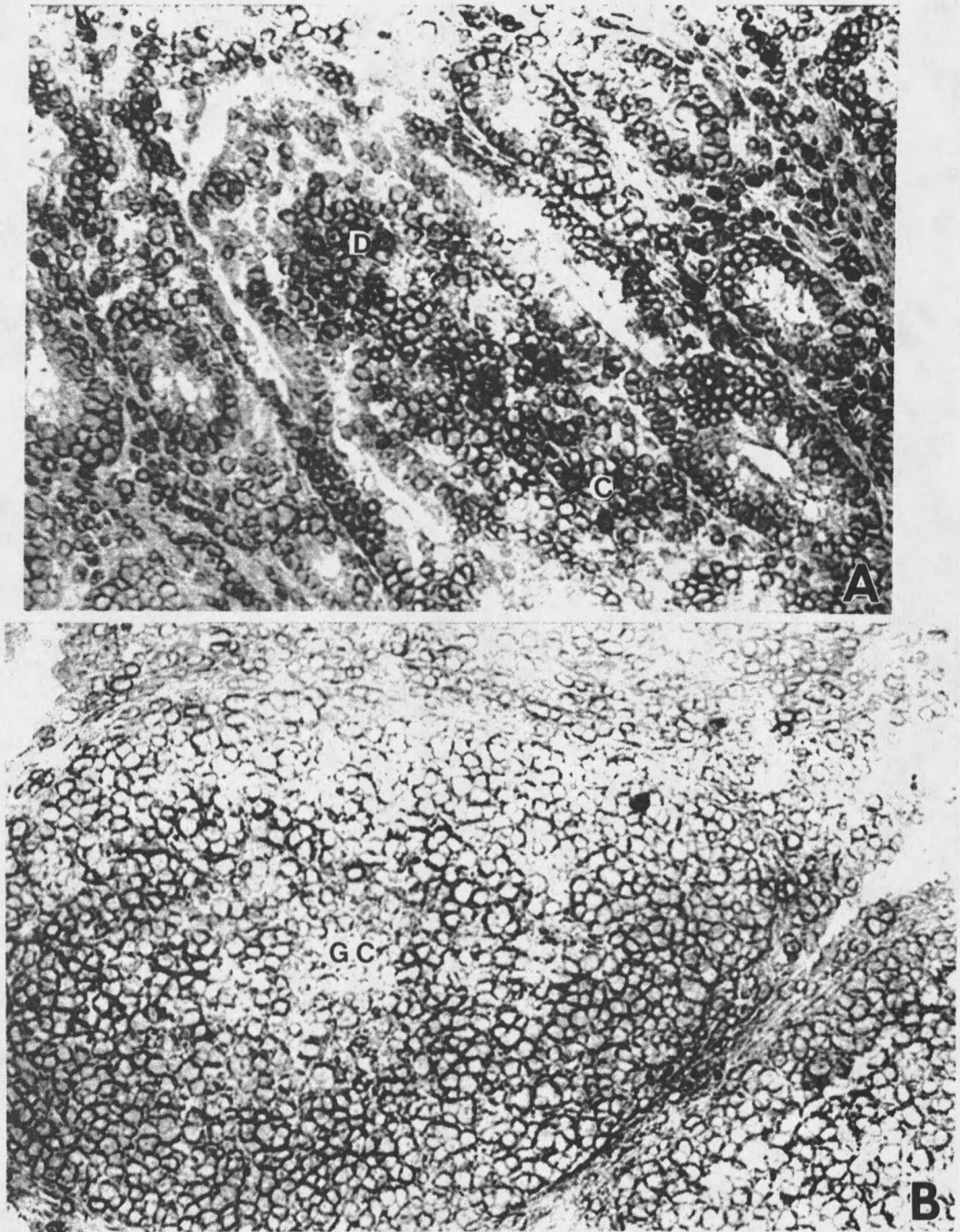


Fig. 23 A. IgG1⁺ cells in dome (D) and corona (C) regions of the Peyer's patch. Positive cells in the lamina propria (L) and intraepithelial (arrows) areas are also shown. X160. Fig. 23 B. In the Peyer's patch follicle, positive cells are on the periphery of a germinal center (GC). X200.

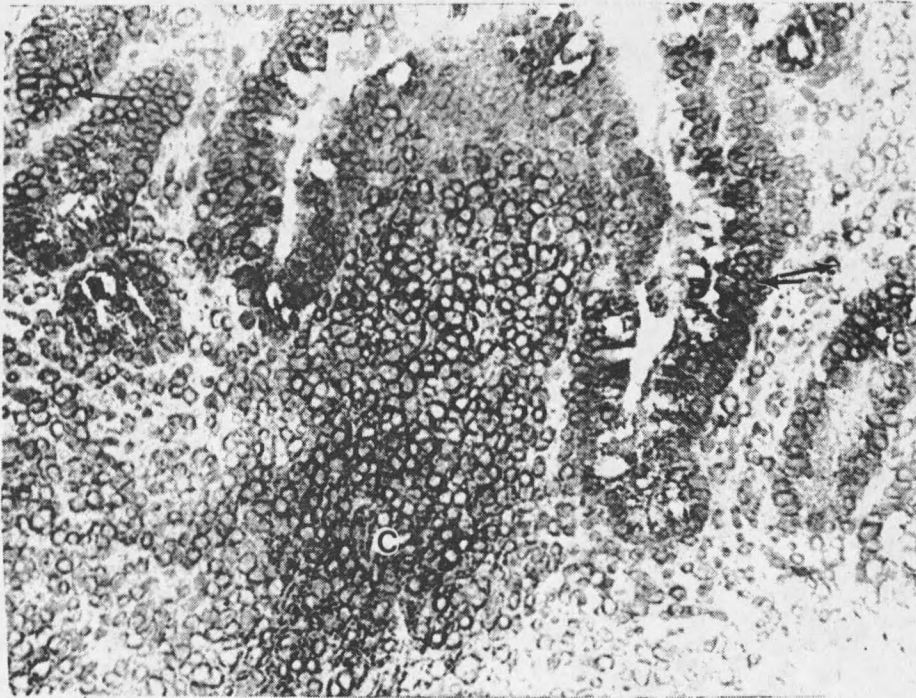


Fig. 24. IgG2⁺ cells in the corona region (C) and dome of a Peyer's patch. Some positive cells are also located in the luminal epithelium (arrows). X160.

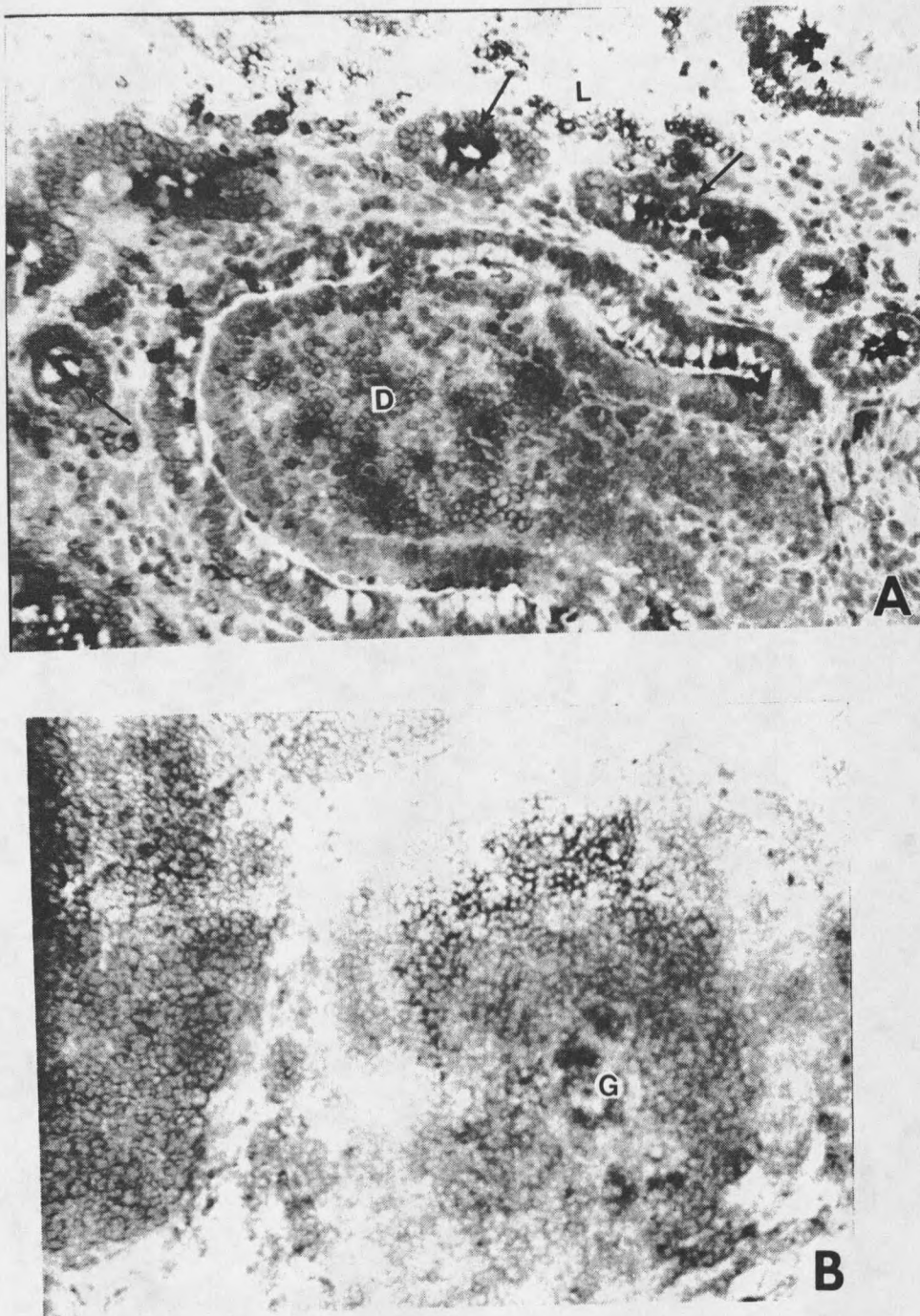


Fig. 25 A. Localization of IgA⁺ cells in a Peyer's patch dome (D) and the lamina propria (L). Note the intensely staining epithelial borders of glandular crypts (arrows). X160. Fig. 25 B. IgA⁺ cells in the peripheral pole of a germinal center (G) in the ileal Peyer's patch. X160.

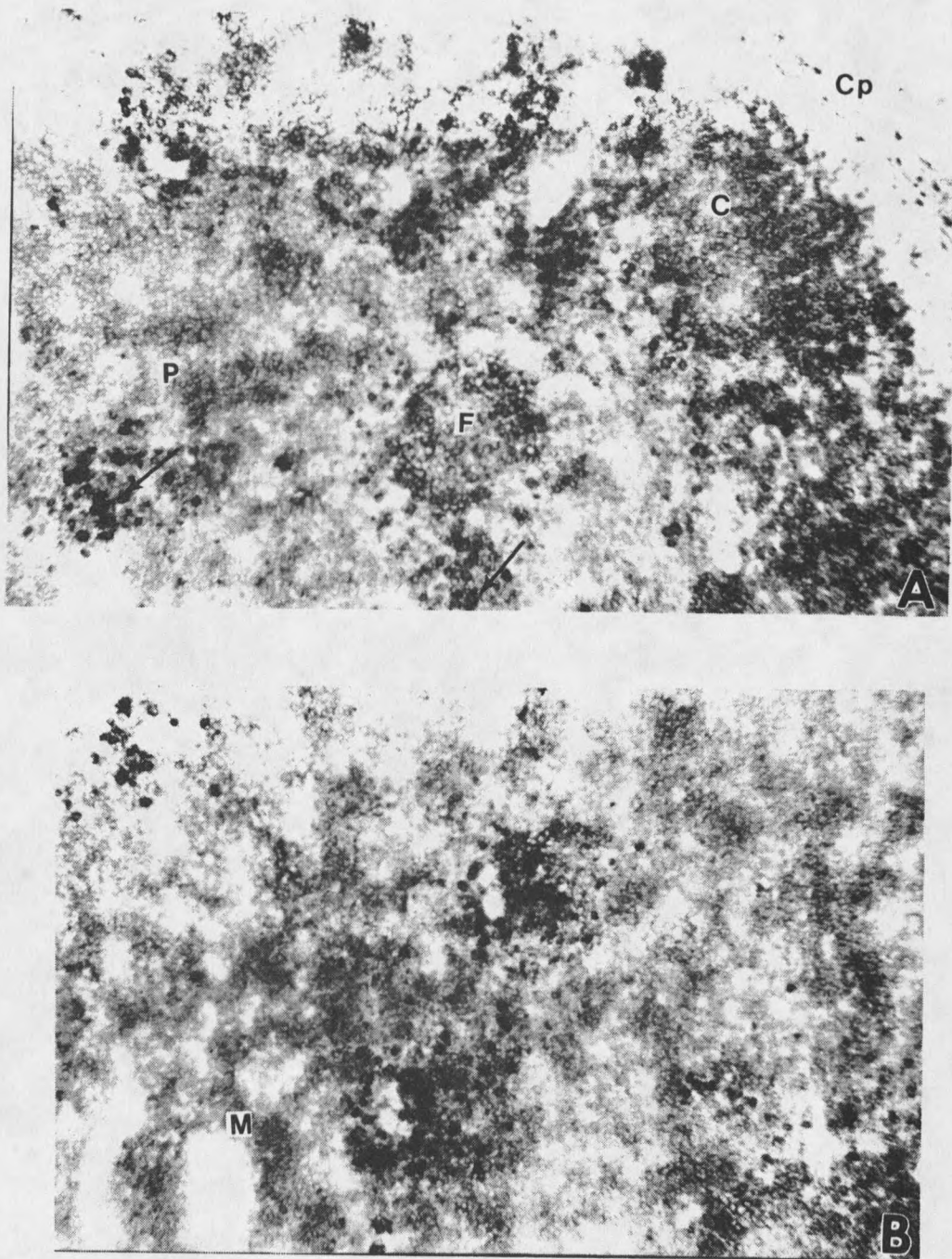


Fig. 26 A. IgM⁺ cells located in the cortex (C), follicles (F) and deep cortex (arrows) of an ileal mesenteric lymph node. The capsule (Cp) is shown at top right. Note absence of positive cells in the T-dependent parafollicular areas (P). X100. Fig. 26 B. IgM⁺ cells in the medullary region (M) of the MLN. X100.

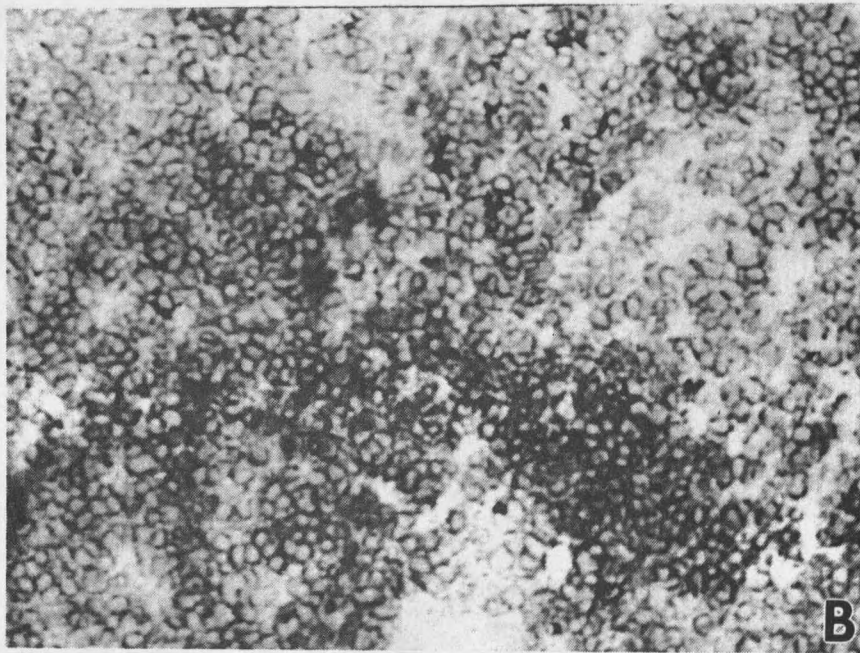
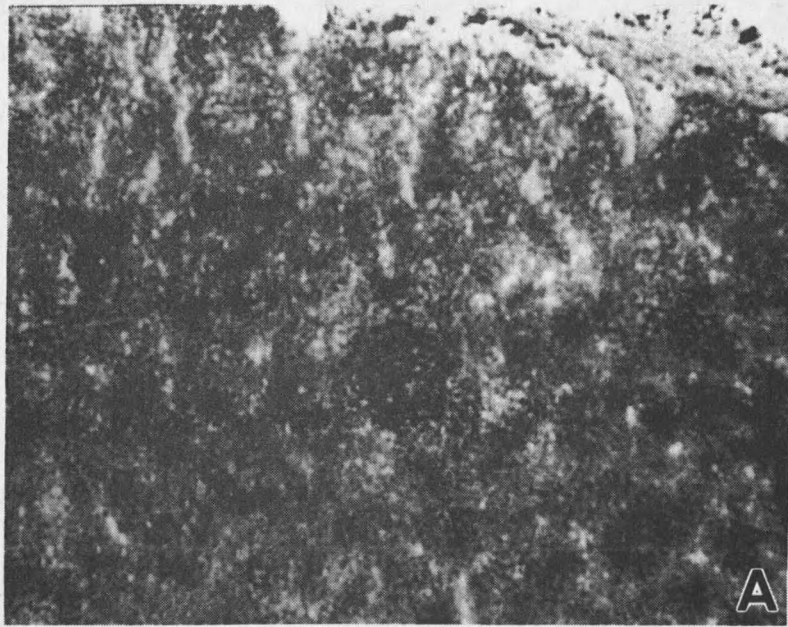


Fig. 27 A. IgG1⁺ cells (arrow) in a secondary follicle in the deep cortex of MLN. X160. Fig. 27 B. IgA⁺ cells (arrows) in the medulla of MLN. X160.

Discussion

Repeated challenge with E. bovis resulted in a significant increase in the frequency of T cells in the MLN, as determined by flow cytometry. The increase was due to an absolute increase in the number of cells expressing the BoT4 phenotype. However, no significant alteration in the frequency of BoT8 cells was observed in any of the tissues studied. In the spleen and ileum, such changes were not apparent in the frequency of any of the T cell markers studied. Similarly, while in all tissues examined a shift favoring relative increases of BoT4⁺ cells occurred, statistically significant differences in the frequency of BoT4⁺ between infected calves were observed only in the MLN. These observations suggest that MLN T lymphocytes may be more susceptible to antigen-induced alterations than those from the ileum and SPL, further implying greater stability of T4:T8 ratios at the latter two sites. Alternatively, there may be preferential accumulation of T4 cells in the MLN. These results may further suggest the relative importance of the MLN, an organ whose afferent traffic partly originates from the intestinal wall, in the expansion of T cells, especially T helper cells, in response to enteric infection with E. bovis. The less dramatic increases in T4:T8 ratios in the gut and spleen may be a further indication of the relative

importance of the T-helper cell in E. bovis infection, a feature also observed in circulating T cells (see Chapter 2).

The marked reduction in the surface expression of BoT8 molecules, was present in splenic, MLN and ileal tissues obtained from infected calves. This finding may indicate the relative numeric stability of the T8 subset; lowered surface expression of the molecule may be a more important functional response of this subset than absolute changes in frequency, a phenomenon that may impact these cells' negative regulatory influence on T helper cells (145, 146). The net effect may, therefore, be a further enhanced functional activity of T helper cells, manifestations of which include enhanced microbicidal capacity of macrophages (87, 88) and increased interferon production by T cells (88, 91). Both manifestations have been proposed as possible effector mechanisms in immune responses to coccidia (87, 88). In vitro mitogen activation of PBM reproduced the effect of lowered surface T8 expression, implying that similar effects are induced by antigen-independent activation.

In the observations reported here, differences between organ compartments in the frequencies of T cells and T4:T8 ratios has been a consistent feature in the tissues examined. In particular, lower T4 relative to T8 values were observed in all calf spleens examined, indicating unique patterns of lymphocyte traffic regulation in this organ. The phenomenon of differential distribution of T cell subsets in lymphoid

organs has been described (32, 118) and it has been proposed that the differences may be due to subset-specific lymphocyte-endothelial cell interaction (32). Immunoperoxidase staining of frozen sections revealed the existence of discrete T cell-dependent areas in the ileal submucosa and Peyer's patches. Aggregates of T cells were located in the interfollicular tissue and the dome of the Peyer's patch. In the lamina propria, T cells were uniformly distributed in the intercryptal regions and infiltrated the intraepithelial tissues. The concentration of T cells in the Peyer's patch domes and interfollicular spaces may facilitate contact between T cells and antigen-presenting M cells that overlie this region (131). The localization of cells expressing the "secondary" Ig isotypes in the dome and corona regions indicates that this site may also be important in T-B cell cooperation and differentiation. Failure to detect the preponderance of any one of these isotypes in the lamina propria indicates that the maturation/differentiation environment of the lamina propria for B cells may not favor the maturation of cells bearing any one Ig isotype. The intense staining of epithelial cells with IgA-specific MAb may nevertheless indicate the relative importance of local secretion of IgA.

Lymphocytes expressing surface Ig isotypes were marginally, but not significantly, altered in tissues from infected calves compared to naive calves. Recirculating

antigen-specific B cells may traffic to other lymphoid and mucosal tissues (131) thereby creating a dilution effect making it less quantitatively apparent in the tissues sampled. The marked increases in IgM and IgG1-bearing cells in circulation may be an indication of the persistence of freshly generated cells of these phenotypes in the peripheral blood.

CHAPTER 4

ANTIGEN-SPECIFIC ANTIBODY ISOTYPES
IN CALVES CHALLENGED WITH EIMERIA BOVISIntroduction

Parasite-specific serum IgG responses to bovine coccidial infections have been demonstrated using indirect fluorescent antibody (44, 49), agglutination (49) and enzyme linked immunosorbent assays (ELISA) (106). These studies showed that parasite specific IgG responses were detectable within 2 weeks after parasite inoculation (PI), peaked at about three weeks and were then sustained for as long as 98 days PI. Serum antibody responses of Ig subclasses or isotypes other than IgG, however, have not been reported for bovine coccidiosis. Certain antigens of E. bovis sporozoites and merozoites (63, 64) have been identified as target molecules of parasite-specific serum IgG. Antigen recognition profiles of specific antibodies belonging to other major serum Ig subclasses, remain undetermined. The identification of major Ig isotypes in the serum of infected animals and their target antigens are crucial to the subsequent identification of potentially protective antibody isotypes.

There is limited information on in vitro antigen-specific antibody synthesis by bovine cells of different tissue origin is available. Studies on the in vitro synthesis of keyhole limpet hemocyanin (KLH)-specific antibody by PBL revealed

significant Ig levels in culture supernatants of cells pulsed with KLH (133, 134). The total IgG1 and IgA synthetic activities of cells of 12 different bovine tissue origins were studied (135). In the presence of pokeweed mitogen, human PBL can be stimulated to synthesize IgM, IgG and IgA (136, 137). Studies on the comparative analysis of specific Ig production by lymphocytes of different tissue origin should provide useful clues on the relative contribution of the tissues examined, to humoral responses in E. bovis infection.

This study was, therefore, undertaken to determine the parasite-specific serum and tissue antibody responses of the immunoglobulins M, G1, G2 and A isotypes in calves repeatedly challenged with E. bovis. The pattern of parasite recognition profiles of these isotypes was also studied.

Materials and Methods

Experimental Infection

Calves were orally inoculated with E. bovis oocysts as described in Chapter 2 above. Serum samples obtained for analysis included those taken prior to and 56 days following the initial oocyst challenge. Identical serum samples were used for immunoblotting. Control sera were obtained from an animal that had been repeatedly infected and used as a source of positive serum, from non-infected calves kept in an isolation unit or from a colostrum-deprived calf.

Cell Culture

Single cell suspensions of lymphocytes from the ileum, MLN and SPL were obtained as described above (see Chapter 3, Materials and Methods). Cells (4×10^6 /ml) were resuspended in RPMI 1640 (supplemented with 10% fetal bovine serum, 50 U/ml penicillin, $50 \mu\text{g}/\text{ml}$ streptomycin, 2mM L-glutamine and 5×10^{-5} 2-mercaptoethanol; C-RPMI) and 0.5ml aliquots were plated onto 24-well polystyrene plates (Corning Glass Works, Corning, NY). The cells were incubated in the presence of 0.5ml ($1 \mu\text{g}$) pokeweed mitogen in C-RPMI or C-RPMI alone, at 37°C , 5% CO_2 -95% air for 7 days, after which the cell suspensions, consisting of cells of identical tissue origin and treatment, were pooled and centrifuged at $1000 \times g$ for 10 min to remove the cells. The supernatants were stored at -20°C until used in ELISAs.

Enzyme-Linked Immunosorbent Assay

For the ELISA, formalin-fixed sporozoites were used as antigen. Sporozoites were isolated and purified as described (87, 117; Chapter 2, Materials and Methods). Briefly, oocysts were treated with sodium hypochlorite (Clorox), rinsed in HBSS and then broken in a motor-driven Teflon-coated tissue grinder. The sporocysts were excysted by treatment with trypsin and sodium taurocholate (see Chapter 2) to release sporozoites, which were then purified by passage over nylon

wool and pelleted by centrifugation. Sporozoites were resuspended in a solution of 0.5% formaldehyde in HBSS at a concentration of 10^7 sz/ml, incubated at room temperature for 5 min, and washed three times in HBSS to remove the formalin. Aliquots of 4×10^6 sz in 0.1M bicarbonate (coating) buffer (pH 9.6) were stored at -20°C .

The basic ELISA protocol used in this study is as described by Richards (132). Sporozoite antigen ($50\mu\text{l}$ /well) was coated onto 96-well polystyrene plates (Immulon 2, Dynatech Laboratories, Inc., Alexandria, VA) and incubated overnight at 37°C . Control wells were coated with $50\mu\text{l}$ ovalbumin in coating buffer ($50\mu\text{g}$ chicken albumin, OVA, Sigma Chemical Co., St Louis, Mo) or coating buffer only. Coated plates were blocked for non-specific activity by treating each well with a 0.1% (v/v) solution of liquid gelatin (Norland Products Inc., New Brunswick, NJ) in 0.15M PBS (pH 7.2); incubating the plates at 37°C for 1 h and then rinsing each well three times with in PBS containing 0.1% Tween 20 (PBS-T). Primary antibody (as diluted serum or undiluted culture supernatant, $50\mu\text{l}$ /well) was incubated in triplicate under identical conditions and the plates washed thrice with PBS-T. Dilutions of the bovine isotype-specific MAb in PBS-T were similarly incubated at the following dilutions: DAS 6 (IgM-specific), 1/4000; DAS 2 (IgG2-specific); DAS 7 (IgA), 1/1000; DAS 17 (IgG1), 1/400. After 3 washes with PBS-T, $50\mu\text{l}$ of 1/4000 alkaline phosphatase-labeled goat anti-mouse IgG

(Boehringer Mannheim Biochemicals, Indianapolis, IN) was added and incubated at 37°C for 1h. The plates were then washed twice with PBS-T and once with distilled water, prior to the addition of 1mg/ml substrate (alkaline phosphatase substrate tablets, Sigma 104, Sigma Chemical Co., St. Louis, Mo) in 0.1M diethanolamine buffer, pH 9.8 (Sigma) and incubated for 2h at 37°C. The enzyme reaction was stopped by adding 50 μ l 3 M NaOH. Absorbance was measured at 405 nm with a micro-ELISA reader (Biotek Instruments Inc., Burlington, VT).

Immunoblotting of Parasite Antigens

Soluble sporozoite antigen for immunoblotting was prepared by subjecting nylon purified sporozoite pellets to 5 freeze-thaw cycles and solubilizing in sample treatment buffer (0.125 M tris-HCl, pH 6.8; 4% SDS, 20% glycerol), adjusted to the equivalent of 2 X 10⁶sz/10 μ l. Merozoites were obtained as described (63). Briefly, sporozoites were used to inoculate the M617 bovine monocyte cell line (Originally obtained from Dr. G. A. Splitter, Department of Veterinary Science, University of Wisconsin-Madison, Madison, WI 53706, and since maintained by continuous cell culture and cryopreservation) and merozoites were harvested from day 14 post-inoculation, washed, pelleted and pooled. Soluble merozoite antigen was prepared as described for sporozoites, adjusted to a final concentration of 5 X 10⁶mz/10 μ l sample buffer.

Antigen preparations (15 μ l/well) and molecular weight standards (Biorad, Richmond, CA) were subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE) in 12.5% slab gels, under non-reducing conditions following described methods (63, 64). Following electrophoresis (30 mA for approximately 2h), the gels were rinsed in two 15-minute changes of transfer buffer (25mM tris, 192mM glycine, 20% v/v methanol) prior to the electrophoretic transfer of parasite proteins in a Bio-Rad Trans-Blot Cell (Biorad, Richmond, CA) to nitrocellulose (NC) paper. In order to visualize the transferred protein bands, the NC paper was washed in a solution containing 7% acetic acid for 5 min and stained for 2 min with Ponceau S solution, after which the NC sheet was cut into 4 mm wide strips. The NC strips, except those with molecular weight standards, were then destained in 7% acetic acid for 5 min and 0.15M PBS pH 7.2 for approximately 30 min and blocked for non-specific binding sites with 10% gamma globulin-free horse serum (Gibco Laboratories, Grand Island, NY) in PBS containing 0.01% thimerosal (Sigma Chemical Co., St. Louis, Mo) at room temperature, for 1h.

After three 10-minute washes in PBS-T, test sera (1/25 in 0.15M PBS, pH 7.2, containing 5% horse serum, .05% Tween 20; C-PBS) were added to the strips and incubated for 1h. The strips were washed in PBS-T and incubated with horse radish peroxidase-labeled goat anti-mouse antibody (1/400 in C-PBS; Boehringer Mannheim Biochemicals) for 1h at room

temperature, washed twice in PBS-T and 0.05M tris-0.2M NaCl (pH 7.4), and incubated at room temperature in peroxidase substrate solution (0.3% w/v 4-chloro-1-naphthol, 16.6% v/v methanol, 8 μ l of 30% H₂O₂ in 0.05M tris-0.2M NaCl). As soon as bands appeared, usually within 30 min, the strips were washed twice in distilled water and air-dried.

Results

Sporozoite-Specific Ig Isotypes in Serum and Culture Supernatant

Specific antibody levels in calves prior to and 49 days after initial challenge with E. bovis oocysts are shown in Table 6. All Ig isotypes studied, except IgG1, were significantly ($p=0.05$) elevated in E. bovis-infected calves compared to non-infected calves. Non-infected control calves exhibited elevated levels earlier which then declined almost 6-fold. A comparison of absorbance (Optical Density) values from both groups with those from colostrum-deprived serum values indicated significantly lower levels of sporozoite-specific IgG1 in the latter. This suggests passive transfer of maternal antibody, particularly IgG1, levels of which waned with time. In the infected group, the post-infection 3-fold increase in serum IgG1 over the pre-infection values represented a 5-fold increase over non-infected, and 20-fold higher over colostrum-deprived serum levels. Elevation of serum IgM suggests the existence of an anamnestic response for

this isotype. The presence of 3-fold higher levels of IgG2 in the serum of a hyperimmunized steer is not clear, but may indicate requirement of this isotype for multiple repeated exposure for its continued elevation. Of interest, is the 6-fold increase of serum IgA in sera from both infected calves and the positive control steer. Elevation of this isotype in serum may suggest that relatively large amounts of monomeric parasite-specific IgA may be transported to the blood prior to complexing with the secretory component and its subsequent secretion across mucosal surfaces. These results suggest the relative quantitative importance of parasite-specific IgG1 and IgM in the humoral response to repeated exposure with E. bovis.

Tissue culture supernatant isotype levels obtained from pokeweed mitogen-activated lymphocytes are summarized in Table 7. The highest levels of parasite-specific Ig isotypes were found in MLN culture supernatants. Although all tissues exhibited increased synthesis of sporozoite-specific isotypes, only IgG2 and IgA are significantly elevated in tissues other than the MLN. As noted in the cytometry data, the frequencies of Ig-bearing cells in the MLN and ileum were comparable, suggesting that the secretory activity of PWM-activated cells in these tissues may be an indicator of the relative predominance of precursor frequency of parasite specific isotypes in the MLN compared to the other tissues. It should be noted, however, that flow cytometric analysis of ileal

suspensions in this study did not distinguish between cell frequencies at the various anatomical regions (the Peyer's patch, lamina propria and intraepithelial area). Hence, the data presented here may be an indication of the overall relative isotype precursor frequencies in the ileum. Similarly, while the observations on in vitro synthesis do not directly address the relative importance of local Ig synthesis versus circulating antibody in E. bovis infection, the relatively higher levels of the various isotypes in the MLN, suggests that soluble antibody synthesized in this tissue into the peripheral circulation may make an important contribution to the overall parasite-specific humoral immunity. This may be especially relevant to IgA and IgM, as both classes are capable of complexing with the secretory component (38) thereby enabling their transport across mucous epithelial surfaces.

Table 6. Sporozoite-specific serum antibody isotypes in calves repeatedly challenged with *Eimeria bovis* oocysts.

A_{405} (Mean $\pm$ SD [*] ; $\times 10^{-3}$)				
Day 0		Day 49		
Infection	+	-	+	-
IgM	285 $\pm$ 87	298 $\pm$ 25	884 $\pm$ 327 ^a [1326 $\pm$ 17]	372 $\pm$ 42 (13 $\pm$ 6)
IgG1	305 $\pm$ 98 ^a	1305 $\pm$ 8	1018 $\pm$ 361 ^a [1322 $\pm$ 4]	190 $\pm$ 83 (44 $\pm$ 24)
IgG2	117 $\pm$ 59	105 $\pm$ 1	260 $\pm$ 40 ^a [676 $\pm$ 5]	160 $\pm$ 43 (28 $\pm$ 9)
IgA	49 $\pm$ 31	24 $\pm$ 8	182 $\pm$ 67 ^a [194 $\pm$ 27]	31 $\pm$ 15 (11 $\pm$ 5)

*Triplicate values from 4 infected and 3 control sera.

Parentheses: [], hyperimmune serum control; (), colostrum-deprived calf serum.

^aSignificantly different from control values ($p=0.05$).

Control wells: OVA-coated wells, 22 $\pm$ 22; secondary antibody only, 20 $\pm$ 4 (Absorbance values pooled for all 4 isotypes).

Table 7. Sporozoite-specific antibody isotypes in supernatant fluid of pokeweed mitogen-activated cells from different tissues.

A_{405} (Mean $\pm$ SD [*] ; $\times 10^{-3}$)						
MLN		ILEUM		SPLN		
Infection	+	-	+	-	+	-
IgM	687 $\pm$ 324 ^a	36 $\pm$ 8	148 $\pm$ 97	31 $\pm$ 20	451 $\pm$ 323	48 $\pm$ 18
IgG1	320 $\pm$ 28 ^a	60 $\pm$ 27	164 $\pm$ 57	89 $\pm$ 23	225 $\pm$ 21	104 $\pm$ 7
IgG2	310 $\pm$ 34 ^a	104 $\pm$ 17	192 $\pm$ 21 ^a	101 $\pm$ 25	315 $\pm$ 57 ^a	132 $\pm$ 10
IgA	538 $\pm$ 36 ^a	77 $\pm$ 28	241 $\pm$ 35 ^a	37 $\pm$ 21	143 $\pm$ 25	46 $\pm$ 8

*Triplicate means from culture supernatants of cells from 4 infected and 2 non infected calves.

^aSignificantly different from control values ($p=0.05$).

Control wells: OVA-coated wells, 22 $\pm$ 7; secondary antibody, 21 $\pm$ 24; supernatants from unstimulated MLN cultures, 41 $\pm$ 10 (Absorbance values pooled for all 4 isotypes).

Immunoblotting

Sporozoite and merozoite proteins electrophoretically transferred onto nitrocellulose were probed with sera from infected calves and the isotypes identified by the relevant anti-isotype MAb. Figure 28 A,B summarizes the binding profiles of the different parasite-specific isotypes. Contrasting patterns of merozoite and sporozoite antigen recognition is detectable among the different isotypes. Several major bands were revealed by the merozoite-specific IgM, most of which were in the high molecular weight (MW) range (66-200 kD). In contrast, IgG1 revealed about 10 bands which belong to this MW range and the rest between 45 and 20kD. Weak signals were revealed by merozoite-specific IgG2 and IgA. However, the merozoite-specific IgA gave stronger signals than the comparable sporozoite-binding IgA. Sporozoite-specific isotypes similarly revealed differences in recognition profiles, the IgG isotypes recognizing more target bands in the low MW ranges. Sporozoite-specific IgG2 signals were more evident than merozoite-specific binding. Sporozoite-specific IgA signals were weak to nondetectable. A number of factors may be responsible for differences in antibody isotype recognition profiles for identical parasite antigens: the relatively lower serum IgG2 and IgA levels as revealed in the ELISA may partially explain the weak signals of these two isotypes. However, the complete absence of

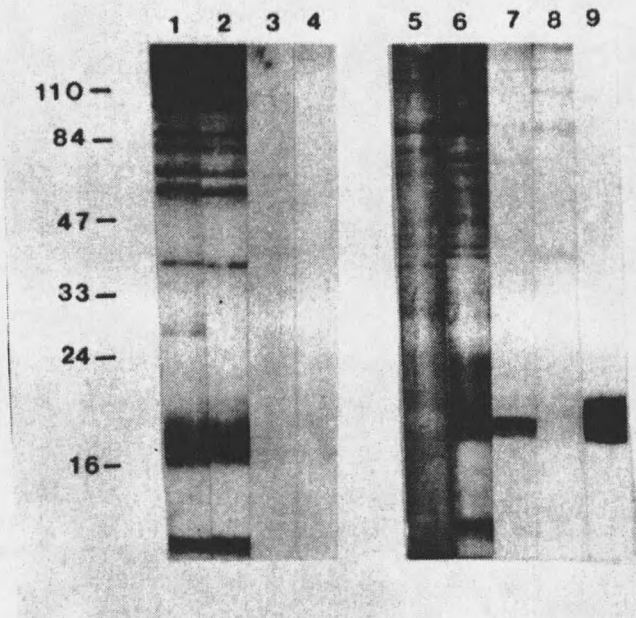


Fig. 28 A. Immunodetection of sporozoite antigens. Protein blots of sporozoites were probed with sera from two calves (lanes 1-4 and 5-8) 49 days after *E. bovis* challenge. The proteins were revealed after incubation with MAb specific for IgM (lanes 1, 5), IgG1 (lanes 2, 6), IgG2 (lanes 3, 7), IgA (lanes 4, 8) and goat anti mouse IgG conjugated to horseradish peroxidase. Lane 9; blot probed with the MAb Ebs 9, specific for a 20 kD sporozoite protein.

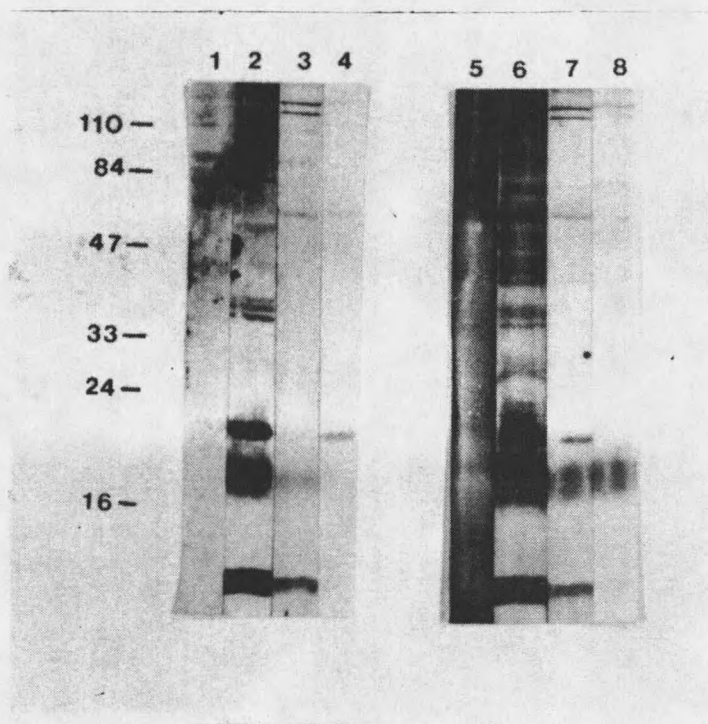


Fig. 28 Continued.

B. Immunodetection of merozoite antigens. Protein blots were probed with sera from the two calves and MAb as in Fig. 28 A. Lane designations 1-8 are identical to those indicated in Fig. 28 A.

binding of some epitopes by either IgM or IgG1 suggests that as more parasite-specific IgG1 cells are generated during secondary and subsequent phases of the immune response, there certain isotype-specific clonotypes may be preferentially generated.

Discussion

Serum antibody responses in calves repeatedly inoculated with E. bovis oocysts resulted in the elevation of all four antibody isotypes as measured by ELISA. Previous studies have concentrated on total serum IgG levels which were also shown to increase in infected calves (44, 49, 106), rodents (53, 55) and sheep (144). Our findings suggest that the increases in IgG levels were mainly due to IgG1. Further, this increase, together with that of IgM, paralleled the increased frequency of circulating B cells expressing these isotypes as demonstrated by flow cytometry. Increases of sporozoite-specific serum IgG2 and IgA, while significant, were of a lower magnitude in contrast to the former two isotypes.

The higher initial levels of IgG1 in control calves relative to colostrum-deprived serum values, suggests the increased levels may have been due to passive colostrum transfer of sporozoite-specific IgG1. These levels declined to near baseline values in sera obtained from control calves about 8 weeks later. The alternative explanation of accidental infection in the control calves is less likely in

view of the housing conditions the calves were kept in. In all calf sera examined, the magnitude of increases in IgG2 and IgA were relatively low, suggesting that the induction of these isotypes may not be significant in the systemic humoral response to the sporozoite stage of the parasite.

Analysis of PWM-activated culture supernatants revealed increased antigen-specific Ig production of all isotypes in the MLN, the highest being IgM and IgA. The spleen exhibited greater variation in IgM production and only IgG2 was significantly increased over values obtained from supernatants of cultures from non-infected control cell cultures. These results may imply that relatively high precursor cell frequencies for most antibody isotypes may be generated in the MLN.

Interestingly, IgA production by MLN cells was greater than two-fold over production by ileal cell suspensions suggesting the possibility of contribution by both local and systemic production to total secreted gut IgA during the parasite-specific responses. It is conceivable, however, that activated precursors may be induced to migrate to the next drainage lymph node site (61), where, upon further encountering antigen are subsequently induced to differentiate into antibody-secreting cells. While this study did not directly address the question of which lymphoid organ may be an enriched source of IgA precursors, both the in vitro Ig synthesis and cytometry data do not suggest that lymphoid

cells of ileal origin are predominantly enriched for IgA precursors. Further, the distribution of membrane Ig-positive cells in the ileum as revealed by immunoperoxidase staining suggest random occurrence of the various phenotypes in the different anatomical sites. Similarly, the association of cells bearing IgG and IgA isotypes with the periphery of Peyer's patch germinal centers suggest the ileum may provide an environment with equal differentiation potential for both secondary Ig classes.

A comparison of the serum isotype binding profiles revealed similarities and differences among the parasite stages and isotypes specific for the same stages. Similarities in recognition patterns of sporozoite and merozoite by identical sera indirectly suggest the presence of shared antigens between the two parasite stages (64), while different patterns indicate the presence of stage-specific antigens. For a given parasite stage, the differences in isotype binding profiles may, in part, be related to the serum levels of an epitope-specific isotype. For instance, the 20 kD protein which has been reported to be immunodominant (14) is recognized by serum IgM and IgG1 but not IgG2 of one animal and by IgG1 and IgG2 but not IgM of another. Differences in serum binding profiles between calves would indicate genetic diversity in antibody recognition. From the ELISA data, sporozoite-specific serum levels for IgG2 and IgA were found to be lower than IgM and IgG1. The fewer bands and lower

intensities of the signals produced by the former two isotypes, therefore, reflect the relatively low specific serum IgG2 and IgA levels. This would in turn suggest that E. bovis may induce the preferential generation of certain antigen-specific isotypes in response to defined sporozoite and merozoite antigens.

CHAPTER 5

INTERACTION BETWEEN EIMERIA BOVIS AND BOVINE TISSUESIntroduction

Parasites belonging to the genus Eimeria have exquisite host species and target tissue specificity (2). The mechanisms governing parasite-host interactions may involve Major Histocompatibility Complex (MHC) gene-related responses, non-MHC genes and the intrinsic ability of parasites to attach to and penetrate permissive host cells. In Escherichia coli, bacterial virulence has been attributed, in part, to adherence of the microbial agent to host cells (138). A recent study (139) revealed significant reduction in the adhesion of E. coli to calf intestines after treatment with bovine plasma glycoprotein glycans. Reduced bacterial adhesion in this system correlated with prevention of enteric disease.

In bovine coccidiosis, sporozoite penetration of a permissive monocyte cell line (M617) is inhibited by sporozoite incubation with a MAb specific for an immunodominant (p20), 20 kDa sporozoite surface protein (13, 14). These findings suggest the potential role of p20 in sporozoite penetration, further implying its involvement in initial surface interaction with monocytes. In addition, recognition of p20 by serum IgG from E. bovis-infected calves (14) may indicate the expression of acquired humoral

resistance in blocking initial surface sporozoite-host cell interactions. Additional evidence for antibody-mediated effector mechanisms targeted at the parasite surface comes from studies demonstrating structural damage on sporozoite surface associated with parasite-specific IgA (15).

Immunoblotting methods have been adapted in other protozoal systems to study molecules involved in interactions between Trypanosoma cruzi and mammalian host cells (17). This approach has led to the identification of 32 and 34 kDa host proteins involved in live and solublized parasite antigen binding.

A major glycoprotein (gp63, ref. 18) and a lipophosphoglycan (LPG, ref. 19) on the surface of Leishmania promastigotes mediate parasite binding to and uptake by macrophages. Both molecules function as ligands for various macrophage receptors, among which the complement receptors CR1 and CR3 appear to be the most important (20, 21). Mice challenged with infective promastigotes are protected by prior immunization with LPG and gp63 peptides (22, 23).

The identification of molecules involved in parasite attachment to and uptake by host cells has, therefore, led to a better understanding of parasite evasion strategies (24) and initiate novel molecular approaches to parasite vaccine strategies.

An ex vivo assay has been used to characterize lymphocyte homing receptor-endothelial cell interactions (140, 141).

This assay has been successfully adapted to study the interaction between Candida albicans and mouse tissues (142). The organism's ex vivo tissue binding patterns mostly correlated with those observed in vivo. This assay was used in this study to characterize the sporozoite-host tissue binding patterns. In addition, a modified immunoblotting procedure (17) was used to partially characterize host enterocyte proteins involved in interactions with sporozoite and merozoite antigens.

Materials and Methods

Ex Vivo Parasite Binding Assay

Tissues of different origins were snap-frozen on dry ice as described (see Chapter 4, Materials and Methods) and 10 μ m thick cryostat sections obtained were used immediately for the binding assay. The technique previously utilized in characterizing lymphocyte-endothelial interaction (140, 141) was used to study E. bovis sporozoite adhesion to various calf tissues. Both crude and nylon wool purified sporozoites were used in the binding assay. The sporozoites were obtained as described (see Chapter 3, Materials and Methods). "Crude" sporozoite preparations were not subjected to nylon wool purification procedures, but were washed in Hank's balanced salt solution after the enzyme excystation stage. For the binding assay, 5×10^5 sporozoites were resuspended in 100 μ l

Dulbecco's Modified Eagle Medium (DMEM, Mediatech, Washington, D. C.) with 10mM HEPES and 5% newborn calf serum (Hyclone, Logan, Utah).

The sporozoite suspension was layered onto the tissue sections at 4°C and constant rotation (70 rpm) for 20 min, after which slides were carefully tilted on absorbent paper to remove unbound parasites. The sections were then fixed in cold 1% glutaraldehyde (Fisher, Fair Lawn, NJ) in Dulbecco phosphate buffered saline, pH 7.2 (Sigma Chemical Co., St. Louis, Mo.) for 30 min, and rinsed several times with cold tap water. Tissue sections were examined by light microscopy for the presence of adhering sporozoites. In order to enumerate tissue binding sporozoites, the sections were stained with hematoxylin and eosin and examined with a Nikon Labophot light microscope. For each region examined, the total number of sporozoites were enumerated in 10 microscopic fields at 400X magnification.

Immunoblotting Procedure

Ileal, cecal and colonic enterocytes were obtained from a 4-week old bull calf as described (129). Briefly, the intestines were thoroughly cleaned with 0.15M phosphate buffered saline (PBS) pH 7.2 to remove ingesta. The washed intestines were stripped of mesenteric tissue, opened longitudinally, and, in order to free epithelial cells from the tissue, 6 to 9-inch segments were incubated with 5mM EDTA in HBSS pH 7.2 for 45min at 37°C, with occasional agitation.

The resultant HBSS, containing an almost pure preparation of intestinal epithelial cells, was pelleted at 1500 rpm for 10 min and washed three times to remove the EDTA.

Thymocyte extracts prepared from single cell suspensions of thymic tissue were used as negative controls. Enterocyte and thymocyte pellets were subjected to 5 freeze-thaw cycles prior to solubilizing in 0.25% octyl glucoside (Sigma), after which the lysates were centrifuged at 3000 rpm for 10 min to remove large tissue aggregates. The supernatant was then centrifuged at 10,000g for 30 min, dialyzed extensively (4 buffer changes over 48 h) against 0.15M PBS, pH 7.2 and protein content determined by the Bradford method according to the manufacturer's instructions.

The tissue extracts were biotinylated as described (143). A solution of 10 mg/ml N-hydroxysuccinimide biotin (NHS biotin, Sigma Chemical Co., St. Louis, Mo) in dimethyl sulfoxide (Sigma) was added to the lysates (1mg/ml protein) in 0.1 M sodium borate buffer, pH 8.8. The biotin ester was added to the protein solution at a ratio of 100 μ g ester per mg protein. The reactants were thoroughly mixed and incubated at room temperature for 4 h, the reaction stopped by adding 20 μ l of 1 M NH_4Cl per 250 μ g ester (RT, 10min). The biotinylated lysates were then extensively dialyzed against 0.15M PBS pH 7.2 and stored at -20°C until further use.

Biotinylated lysates were used to probe sporozoite and merozoite antigens. Solubilized parasite antigens were

subjected to SDS-PAGE and electrophoretically transferred onto nitrocellulose (NC) as described (see Chapter 4, Materials and Methods). Blocking for non-specific binding was accomplished by incubating the NC strips with 10% gamma globulin-free horse serum (GGF-HS, Gibco) in 0.15M PBS, pH 7.2, 0.01% thimerosal (Sigma) for 1h at room temperature. The strips were then washed 3 times in PBS-Tween 20 (5 min. per wash).

Two ml of the tissue extracts (100 μ g/ml protein) or immune serum (diluted 1/20) in PBS/5% GGF-HS were incubated with the strips for 1 h at room temperature, prior to washing as above and further incubation with 1/2000 streptavidin peroxidase conjugate (TAGO, Inc., Burlingame, CA) in PBS/5% GGF-HS for 1h at room temperature. The immune serum was obtained from a steer that had been repeatedly exposed to large doses (10^6) of oocysts and kept as a source of immune serum and sensitized blood lymphocytes. The strips were washed once in PBS-Tween 20, twice in 0.05M tris-0.2M NaCl (pH 7.4), then incubated in peroxidase substrate solution (0.3% w/v 4-chloro-1-naphthol, 16.6% v/v methanol, 8 μ l of 30% H₂O₂ in 0.05M tris-0.2M NaCl). Strips incubated with the serum were in addition incubated with a 1/400 dilution of a mouse MAb specific for bovine light chain (DAS 9; Ultimate Conceptions, Millers Falls, MA) for 1h at room temperature, prior to incubation with the peroxidase substrate solution for about 30 min to reveal parasite-bound tissue extracts or bound antibody.

Results

The quantitative tissue binding characteristics of two batches of sporozoites to 11 calf tissues are shown in Table 7. In general, binding to gastro-intestinal tissue is much higher in epithelial than in non-epithelial regions (lamina propria, Peyer's patch) of the same tissues. Similarly, sporozoite binding to tissues of non-gut origin is significantly lower than in gut epithelia (Figs. 29-32). These trends hold for both sets of sporozoite (purified versus crude) preparations tested. In epithelial sites with the highest numbers of adherent sporozoites, the purified sporozoite preparations revealed greater numbers of sporozoite adherence than their crude counterparts. The presence of contaminants and other components (oocysts, oocyst walls, sporocysts) in the crude sporozoite fractions may have partial inhibitory or competitive effects on sporozoite binding. Adherence patterns of oocyst and sporocyst contaminants closely paralleled those exhibited by sporozoites. These structures were, however, not retained during the staining procedure and could, therefore, not be revealed in the photographs. The presence of large numbers of adherent sporozoites in the duodenum, but not jejunum, is intriguing in view of no known sporozoite invasion of the former site in natural coccidial infections and that sporozoite release

occurs in the lower intestinal tract. The possible significance of this phenomenon in the disease is not clear.

Table 8. Binding of Eimeria bovis sporozoites to different bovine tissues.

Tissue	No. adherent Sporozoites	
	Purified ^a	Crude
Duodenum		
Epithelium	2540	498 (39)
Lamina propria	23	54 (8)
Jejunum		
Epithelium	130	175 (107)
Lamina propria	116	65 (35)
Ileum		
Epithelium	3276	1178 (76)
Lamina propria	340	140 (119)
Cecum		
Epithelium	2330	516 (82)
Lamina propria	225	70 (52)
Colon		
Epithelium	402	536 (187)
Lamina propria	9	55 (64)
Peyer's Patch	11	23 (10)
Mesenteric Lymph Node	9	7 (4)
Spleen	102	68 (35)
Thymus	7	7 (8)
Liver	5	21 (21)

^aTotal number of sporozoites in 10 microscopic fields at 400X magnification.

^bMean value (parentheses, standard deviation) from 2 or 3 independent experiments each representing all sporozoites in 10 microscopic fields at 400X magnification.

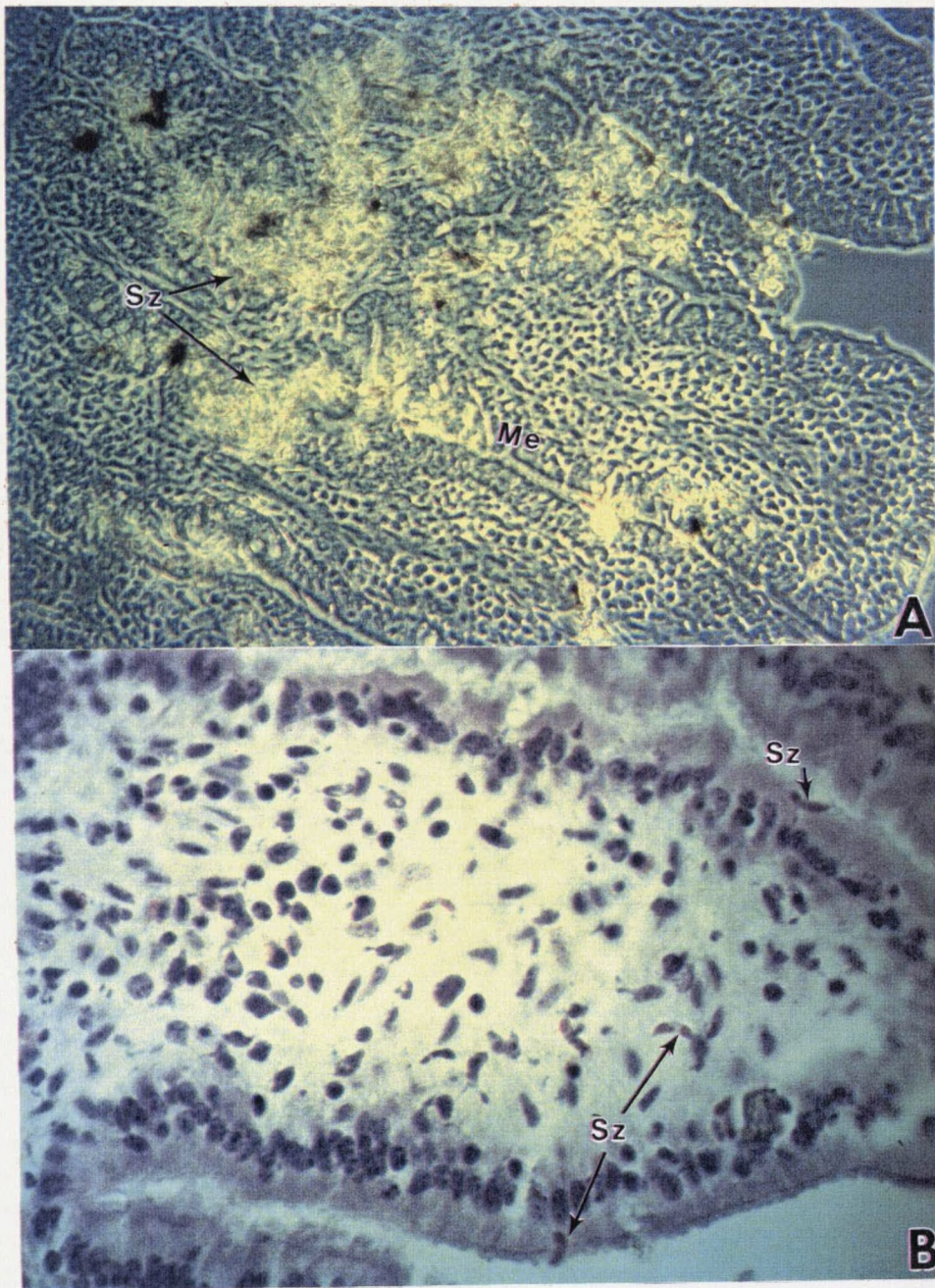


Fig. 29 A. Sporozoites (Sz, arrows) bound to ileal mucosal epithelial surfaces (Me). The $10\mu\text{m}$ thick section is shown with adherent sporozoites prior to staining (X200). Fig. 29 B. Bound sporozoites (Sz) in a section of the ileum after staining with H&E (X400).

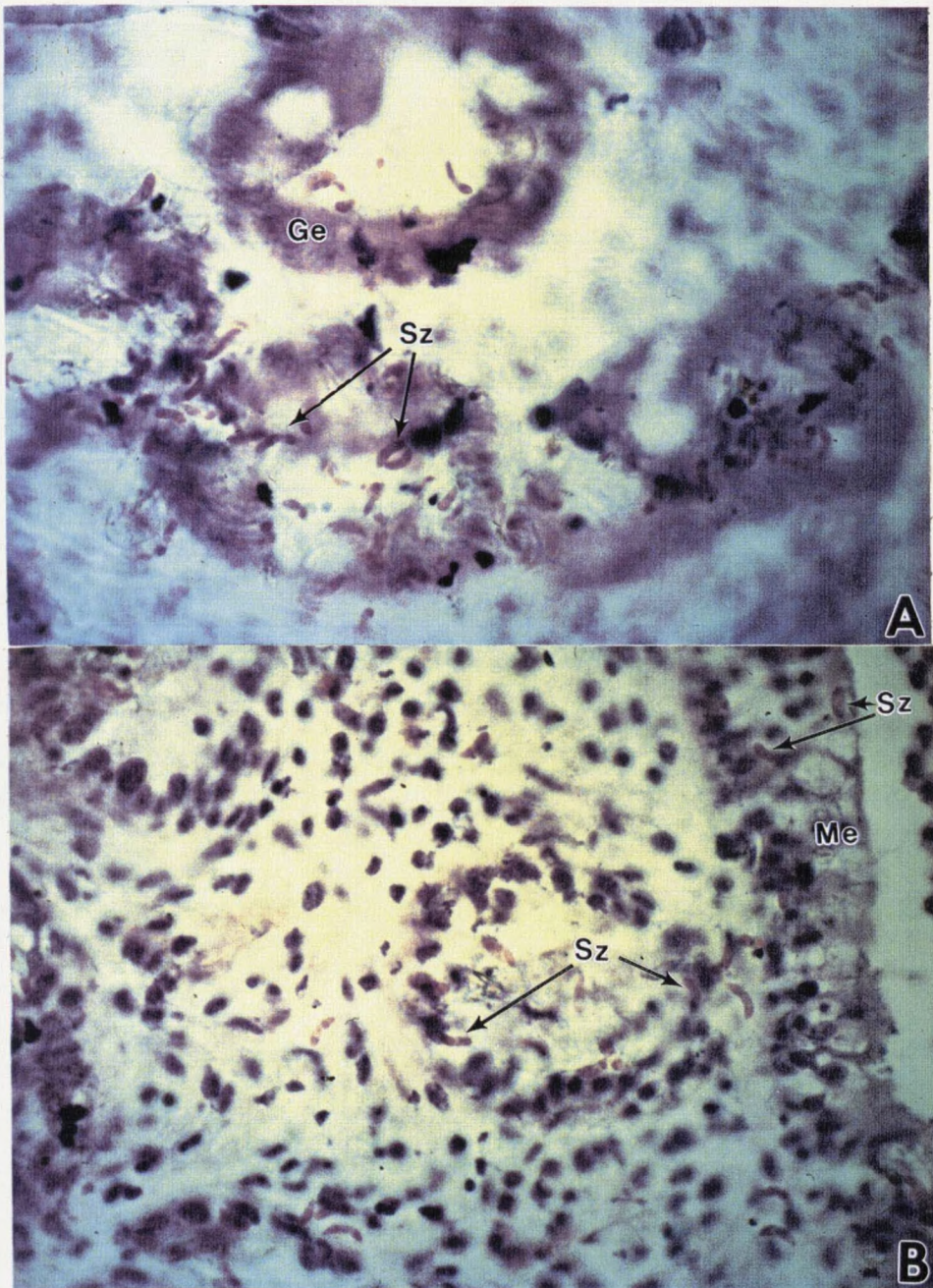


Fig. 30 A and B. Adherent sporozoites (Sz, arrows) on glandular (Ge) and (B) mucosal epithelial (Me) surfaces in sections of the cecum stained with H&E. (X400).

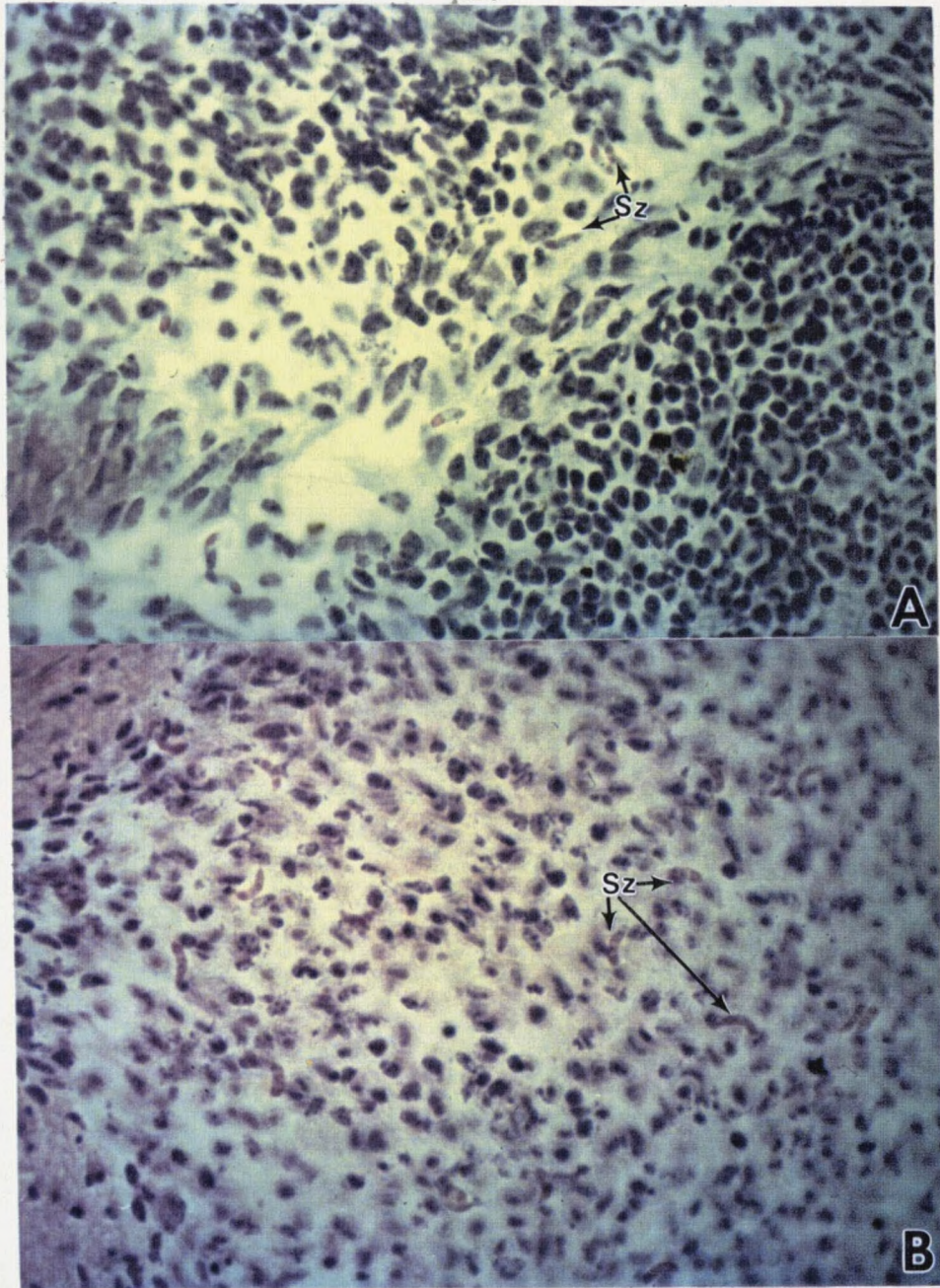


Fig. 31. Sporozoites (Sz, arrows) binding to spleen (A) and thymus (B). Fewer parasites were observed binding to these tissues than in gut tissue. After the binding assay, the tissues were stained with H&E. (X400).

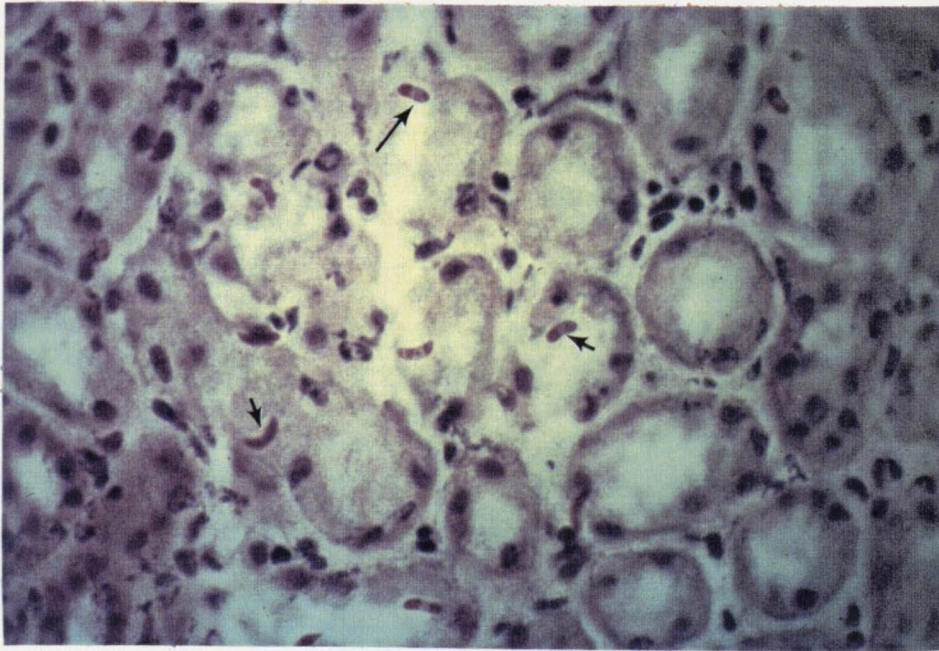


Fig. 32. Eimeria bovis sporozoites (arrows) binding to renal tissue are mostly associated with tubular epithelium (X400).

In the modified blotting procedure using small intestinal (SI), large intestinal (LI), thymic lysates and serum from an immunized steer to probe sporozoite and merozoite antigens transferred onto NC strips, clear differences in the binding patterns are evident (Fig. 33). The lysate from the large intestine recognized a >110 kDa sporozoite determinant that was weakly revealed by the large intestinal extract (lane 2). Similarly, a contrasting recognition pattern is revealed by the immune serum probed with an anti bovine light chain monoclonal antibody (lane 3), suggesting that some parasite antigens involved in adhesion to host tissues may not be recognized by serum antibodies. Differences were also evident in the recognition patterns of merozoites, the LI binding a protein about 70 kDa not recognized by the SI (lane 6) or the immune serum (lane 7). Large intestinal lysate recognition of fewer merozoite determinants than either sporozoite or those revealed by immune serum and the recognition of a unique protein by the large intestinal extract, suggest that the adhesion between merozoites and the large intestine may be more specific than that exhibited by sporozoites for intestinal epithelium. The thymic lysates revealed weak to indiscernible signals at the equivalent protein concentrations (lanes 4 and 8). These results are consistent with the sporozoite adhesion patterns observed in the ex vivo binding assay.

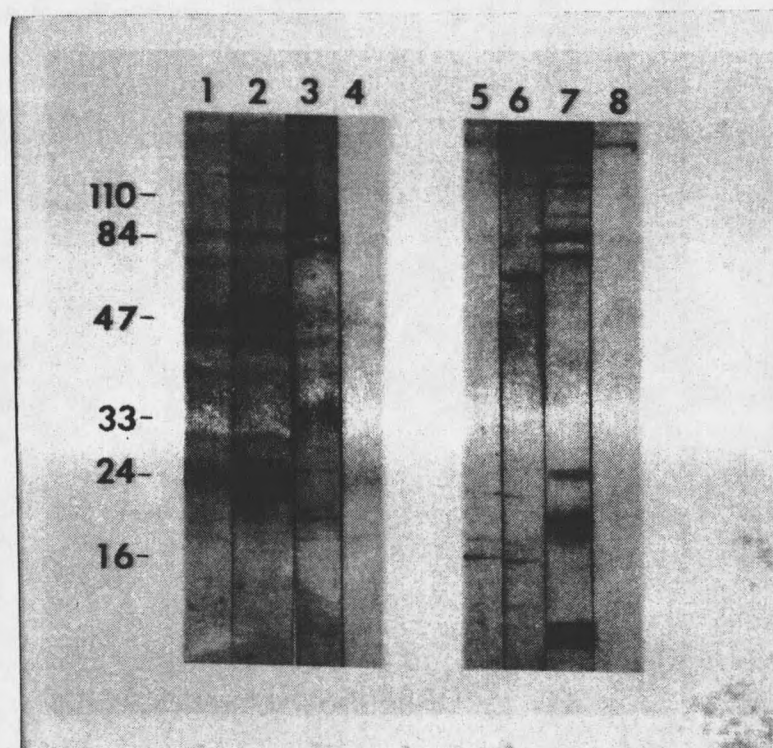


Fig. 33. Protein binding assay. Protein blots from *E. bovis* sporozoites (lanes 1-4) and merozoites probed with biotinylated lysates from bovine small intestine (lanes 1, 5), large intestine (lanes 2, 6), immune serum (lanes 3, 7) and thymic lysates (lanes 4, 8).

Discussion

Results from the ex vivo sporozoite binding assay are consistent with the known in vivo development of E. bovis. There was preferential sporozoite adhesion to the small intestine, compared to other tissues. Further, comparative analysis of binding patterns between the epithelial, lamina propria and lymphoid areas of the gut revealed much higher binding in glandular epithelial areas, indicating that sporozoites may first contact host tissue at this site. Sporozoite binding to the duodenum suggests shared cognate proteins with those of the ileum. In natural infections, however, this is unlikely to be of significance in light of sporozoite release occurring further down the alimentary tract. In addition, the parallel binding of oocysts and sporocysts in identical regions of the gut, interactions between these stages and mucous surfaces may partially immobilize them and allow their exposure to gastro-intestinal enzymes that release them.

Although the protein binding data revealed complex intestinal tissue molecules involved in sporozoite and merozoite binding, the lysates did reveal unique tissue lysate binding to parasite proteins, suggesting tissue or site-related differences in the molecules involved in adhesion. These observations reflect trends seen in the ex vivo binding assay, given the poor binding revealed by the single non gut

tissue (thymic extract) control examined. Binding of large but not small intestinal lysates to merozoite antigens is in concordance with the known site for in vivo E. bovis development for this parasite stage. The binding patterns revealed by lysates used to probe the two parasite stages revealed fewer merozoite molecules involved in binding to host tissues than sporozoites, further suggesting potential differences in tissue-binding mechanisms of the two parasite stages. These assays provide novel approaches to the identification of molecules that participate in parasite attachment to host cells. Furthermore, the identification of parasite proteins that bind host tissues and their purification should provide an opportunity for evaluating them as immunogens in the generation of high affinity antibodies that could block this interaction. Interference with adhesive parasite-host interactions could potentially block, or significantly lessen, the pathogenic effects of invasive stages in the parasite cycle.

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