



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:

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 BoTB. 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.

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.

The *ex vivo* binding of sporozoites to various tissue sections revealed preferential attachment to gut tissue and that gut epithelial binding was higher than in the subepithelial microenvironment. These findings were consistent with observations of a modified blotting procedure in which biotinylated enterocyte protein extracts exhibited preferential binding to sporozoites and merozoite antigens over thymic extracts.

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I dedicate this dissertation to my children, Ageel, Nafisa, Aisha, and my mother, Khadija.

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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 propria 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. vermiciformis* 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. falciiformis (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. falciiformis-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 $4^\circ C$ 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 Na₂HPO₄·2H₂O, 0.69g NaH₂PO₄·H₂O, 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 10⁶ 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 100μl 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 50μl 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, 50μl, 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)

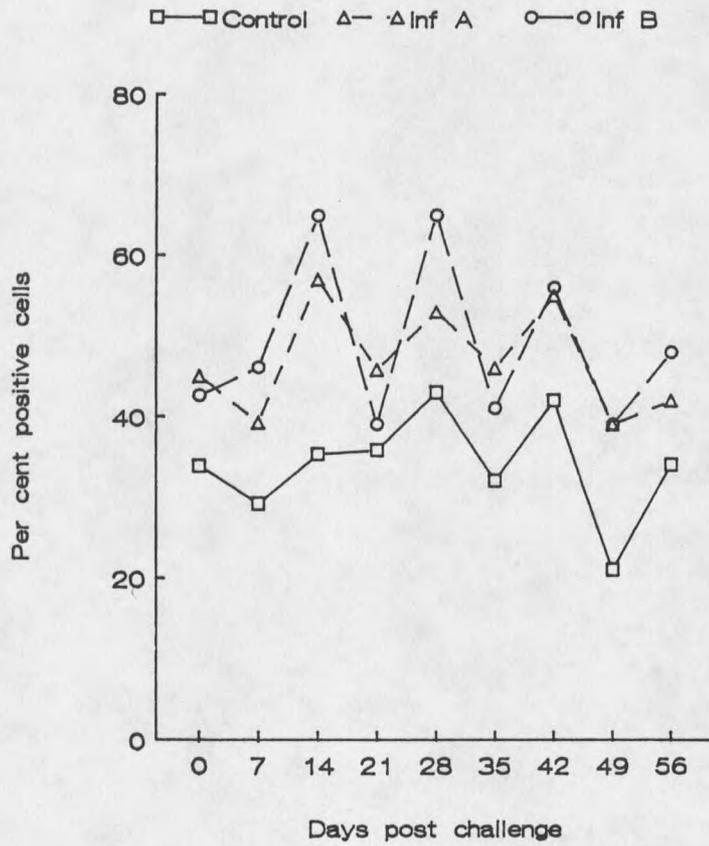


Fig. 1. Sequential alterations in the frequency of circulating T cells in calves challenged with 10^5 E. bovis oocysts.

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