Characterization and effects of monoclonal and polyclonal IgG antibodies on in vitro cell penetration by Eimeria bovis
by William Michael Whitmire

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 effects and localization of parasite-specific monoclonal (MAb), antiserum (AS) or immune serum (IS) antibodies on sporozoites of Eimeria bovis were examined in an in vitro system using phase-contrast and fluorescence light microscopy as well as immunoelectron microscopy (IEM). Twelve MAbs that were elicited against sporozoites, demonstrated at least 4 different fluorescence patterns by indirect fluorescent antibody (IFA) assays on acetone-fixed sporozoites. Three of these MAbs cross-reacted with acetone-fixed merozoites but only 2 MAbs, EbS9 and EbS11, localized specifically on the plasmalemma of glutaraldehyde-postfixed sporozoites by IFA or IEM. AS and IS parasite-specific antibodies were found by IEM to react with the inner and outer surfaces of the inner layer of the oocyst wall, inner surface of the sporocyst wall, outer surface of the sporocyst residuum and the plasmalemma of glutaraldehyde-prefixed sporozoites. Upon exposure to AS or IS, live sporozoites capped and shed immune complexes at their posterior ends. In contrast, live sporozoites exposed to EbS11 or EbS9 did not cap or shed immune complexes. Pretreatment of live sporozoites with EbS9 or EbS11 resulted in a 79 and 73% decrease, respectively, in penetration of cultured Madin-Darby bovine kidney (MDBK) cells. No significant differences in cell penetration occurred in MDBK cells inoculated with sporozoites that had been pretreated with other MAbs, AS, or IS. None of the MAbs or AS interfered with the ability of sporozoites to undergo merogony in M617 cells. Pretreatment with IS abolished the ability of sporozoites to develop in M617 cells. However, this effect may have been due to factors (lymphokines) other than parasite-specific antibodies that may have been present in IS. Immunodetection of nonreduced sporozoite antigens on western blots demonstrated that both EbS9 and EbS11 reacted with the same relative molecular weight 20,000 (P20) protein band. AS and IS also reacted intensely with P 2 0 indicating that P 2 0 may be immunologicalIy important.

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ABSTRACT

The effects and localization of parasite-specific monoclonal (MAb), antiserum (AS) or immune serum (IS) antibodies on sporozoites of Eimeria bovis were examined in an in vitro system using phase-contrast and fluorescence light microscopy as well as immunoelectron microscopy (IEM). Twelve MAbs that were elicited against sporozoites, demonstrated at least 4 different fluorescence patterns by indirect fluorescent antibody (IFA) assays on acetone-fixed sporozoites. Three of these MAbs cross-reacted with acetone-fixed merozoites but only 2 MAbs, EbS9 and EbS11, localized specifically on the plasmalemma of glutaraldehyde-postfixed sporozoites by IFA or IEM. AS and IS parasite-specific antibodies were found by IEM to react with the inner and outer surfaces of the inner layer of the oocyst wall, inner surface of the sporocyst wall, outer surface of the sporocyst residuum and the plasmalemma of glutaraldehyde-prefixed sporozoites. Upon exposure to AS or IS, live sporozoites capped and shed immune complexes at their posterior ends. In contrast, live sporozoites exposed to EbS11 or EbS9 did not cap or shed immune complexes. Pretreatment of live sporozoites with EbS9 or EbS11 resulted in a 79 and 73% decrease, respectively, in penetration of cultured Madin-Darby bovine kidney (MDBK) cells. No significant differences in cell penetration occurred in MDBK cells inoculated with sporozoites that had been pretreated with other MAbs, AS, or IS. None of the MAbs or AS interfered with the ability of sporozoites to undergo merogony in M617 cells. Pretreatment with IS abolished the ability of sporozoites to develop in M617 cells. However, this effect may have been due to factors (lymphokines) other than parasite-specific antibodies that may have been present in IS. Immunodetection of nonreduced sporozoite antigens on western blots demonstrated that both EbS9 and EbS11 reacted with the same relative molecular weight 20,000 (P20) protein band. AS and IS also reacted intensely with P20 indicating that P20 may be immunologically important.

INTRODUCTION

Taxonomic Classification of Eimeria bovis

Subkingdom: PROTOZOA Goldfuss, 1818 emended Siebold, 1845

Phylum: APICOMPLEXA Levine, 1970

Class: SPOROZOASIDA Leuckart, 1879

Subclass: COCCIDIASINA Leuckart, 1879

Order: EUCOCCIDIORIDA Leger and Duboscq, 1910

Suborder: EIMERIORINA Leger, 1911

Family: EIMERIIDAE Minchin, 1903

Genus: EIMERIA Schneider, 1875

Species: BOVIS Fiebiger, 1912

History

Since almost all protozoans are of microscopic dimensions (certain free living forms are exceptions), it was not until the invention of the microscope that they were first seen. In a historical review of the coccidia, Levine (1973) states that in 1674 Leeuwenhoek saw oocysts of Eimeria stiedai in the bile ducts of a rabbit (61). This was the first parasitic protozoan ever to be seen. It was not until 1839, more than 150 years later, that Hake described this parasite in which he thought that the oocysts were pus globules originating from liver carcinoma in

rabbits (61). An additional 50 years were required before the basic eimerian life cycle was described. Kauffman in 1847, described sporulation within the oocyst followed by the delineation of the endogenous life cycle of Gregarina falciformis in mice by Eimer in 1870 (61). Eimer believed that oocysts spread the infection from one animal to another and that the parasite multiplied by schizogony (merogony) in an endogenous cycle. This organism was later renamed Eimeria falciformis by Schneider in 1875 and became the type species of the genus Eimeria (61).

According to Levine (1973) Eimer's theory, although correct, was contested by Schneider in 1892 as well as Labbe in 1896 and others who thought that two different genera were responsible for the different parasite stages (61). Meanwhile, L. Pfeiffer and R. Pfeiffer in the early 1890s suggested that the parasite possesses alternation of generations. They determined that Eimeria stiedai in the liver of rabbits first multiplied and then produced oocysts. This idea was criticized as well, until proven correct (61). The entire life cycle of Eimeria falciformis was described by Schuberg in 1895, confirming the works of Eimer and Pfeiffer and Pfeiffer (61). Members of the genus Eimeria are monoxenous obligate intracellular protozoan parasites which possess an alternation of asexual and sexual generations. Presently, this genus contains over a thousand species which occur mainly in vertebrate hosts (62).

General

Coccidiosis is a complex intestinal disease that occurs in various species of animals, including chickens, turkeys, rabbits, sheep and cattle (78). However, the greatest economic impact is probably sustained by the cattle and poultry industries of the world. In 1972, Fitzgerald estimated that bovine coccidiosis caused an annual world-wide monetary loss of 472 million dollars (30). Other investigators stated that in the United States alone, 60 to 120 million dollars are lost each year by the poultry industry (98, 106). This sum does not include the cost of anticoccidial drugs which amount to another 35 million dollars per year (106).

The etiologic agents of coccidiosis are members of the genus _Eimeria_. These organisms display a high degree of host specificity and generally infect digestive tract tissues which may lead to diarrhea, destruction of intestinal epithelium, weakness, weight loss, retardation of growth and death (49, 64, 71, 76).

Outbreaks of coccidiosis generally result from the abnormal crowding of host species into a limited area. Under these circumstances the host may acquire a sufficient quantity of oocysts to produce clinical symptoms (61). The severity of the infection is dependent on the numbers of oocysts ingested as well as any stressful situations that

may be experienced by infected animals. However, the potential for the multiplicity of these parasites is limited since the infection is terminated after the completion of the life cycle (35).

Although there are several species of _Eimeria_ that infect cattle, only two species, _E._ _bovis_ and _E._ _zuernii_, are known to produce clinical disease (35, 78). Experimental infections with _E._ _bovis_ are relatively easy to produce whereas experimental infections with _E._ _zuernii_ have proven inordinately difficult to establish. For this reason, most experimental investigations concerning bovine coccidiosis have dealt with infections by _E._ _bovis_. _Eimeria_ _bovis_ has been reported as the most frequent cause of bovine coccidiosis in the United States and other parts of the world while _E._ _zuernii_ is largely responsible for coc-cidiosis of cattle in Canada, Hungary and central Europe (62, 78).

One of the most noticeable clinical features of bovine coccidiosis is severe hemorrhagic diarrhea accompanied by rectal tenesmus, which occasionally results in rectal prolapse (78). Animals may become markedly dehydrated, anemic and emaciated due to the continued loss of body fluids and the onset of anorexia. During this period, partial paralysis of the anal sphincter allows for the incomplete closure of the anus (78). As the disease progresses, an increased respiration rate along with a low

grade fever as well as blood clots and mucous shreds in liquid feces may also be present. Recovery depends on the severity of the infection. However, if infected animals are unable to stand after exhibiting the aforementioned symptoms, the prognosis is poor with little hope of recovery (78). Moreover, the effects of the disease may decrease the market value of surviving animals by causing retardation of growth. For example, Hammond (1964) estimated in 1962 that 90% of all calves in the United States were infected which would result in an average loss of 75 cents per head on all calves less than a year old (35). The general debility of coccidiosis may also render surviving animals susceptible to other pathogens. At the present time, there are no vaccines or suitable preventative measures for bovine coccidiosis.

Life Cycle

The typical life cycle of an eimerian includes endogenous stages inside the host as well as exogenous stages occurring outside of the host. _Eimeria_ species have oocysts that contain 4 sporocysts, each with 2 sporozoites (61). Infection with E. _bovis_ is initiated by the ingestion of sporulated oocysts by cattle (_Bos_ spp.). Upon exposure to carbon dioxide, trypsin and bile in the intestinal tract, sporozoites of E. _bovis_ excyst from oocysts, pass through the ileal intestinal epithelium (36, 66) and penetrate

endothelial cells of the central lacteals (38) where they
undergo asexual reproduction by a process called merogony
(schizogony) to form first-generation merozoites. Mature
meronts (schizonts) average about 300 um (micrometer) by 200
um and contain about 120,000 merozoites (36). Meronts reach
maturity about 14 or 15 days after ingestion of sporulated
oocysts (36). First-generation merozoites presumably
escape into the lumen of the small intestine or travel via
the blood stream to the large intestine and cecum where they
penetrate glandular epithelial cells and undergo develop-
ment to second-generation meronts (36). Second-generation
meronts are relatively small (about 10 um in diameter),
reach maturity in approximately 1 1/2 to 2 days and contain
30 to 36 second-generation merozoites (15). Second-
generation merozoites enter adjacent epithelial cells and
differentiate into micro- and macrogamonts. Each microgamont
gives rise to about 50 motile flagellated microgametes,
whereas each macrogamont develops into a single large
gamont. Microgametes escape from host cells and penetrate
other cells harboring macrogamonts (36, 38). Fertilization
presumably results in the formation of a zygote which
subsequently surrounds itself with an oocyst wall via wall
forming bodies which are present in the mature macrogamont
(36). After the oocyst wall has been completed, oocysts are
discharged into the lumen of the large intestine and cecum
and passed unsporulated in the host feces (35). Upon

exposure to atmospheric oxygen oocysts undergo sporulation to form 4 sporocysts each with 2 sporozoites. Sporulated oocysts are infective to the appropriate, susceptible host. Although oocysts are resistant to many environmental conditions, they are adversely affected by freezing and desiccation (36).

The discharge of E. bovis oocysts begins at about 18 days after ingestion of oocysts by the host followed by peak oocyst passage 2 or 3 days later. However, the patency period lasts for about 2 weeks. Oocysts cause destruction of host enterocytes which if severe enough may result in the clinical symptom of hemorrhagic diarrhea.

In Vitro Cell Penetration and Development

Penetration and development within host cells are 2 crucial events in the life cycles of coccidian parasites. Sporozoites, merozoites and microgametes must actively penetrate host cells in order to ensure completion of the life cycle.

Because coccidian sporozoites and merozoites possess an apical complex at their anterior ends and since motility and host cell penetration occur by means of the anterior end, it has been assumed that the apical complex functions as a cell-penetrating organelle (62, 107). Ultrastructurally, the apical complex consists of 2 apical rings, 2 polar rings, micronemes, rhoptries, about 22 subpellicular

microtubules and a conoid (107). Although there is much speculation, the role of each component of the apical complex during cell penetration is still not known.

The conoid has been observed to be extended, distended, retracted and inserted repeatedly and moved laterally as well as remaining unchanged during in vitro cell penetration (27, 44, 85). Some investigators have suggested that rhoptries play an active role in cell penetration by secreting lytic enzymes or other substances (27, 75). Lycke and Norrby (69) and Lycke et al. (70) discovered a penetration enhancing factor isolated from lysed Toxoplasma gondii tachyzoites which increased the virulence of T. gondii in mice and the extent of in vitro penetration by intact tachyzoites. This factor may be lysosomal in origin since tachyzoites exhibited fewer lysosomes after completion of host cell penetration (77). In some species of coccidia, rhoptries have been found to be empty or diminished in size in zoites during or soon after host cell entry indicating that rhoptries may secrete a substance which aids penetration (46, 47, 57, 75). However, rhoptries may also remain unchanged in size or density during penetration (85).

Toxoplasma gondii, Plasmodium spp., Isospora canis, as well as E. magna have been reported to enter host cells without disrupting host cell membranes by merely invaginating the host cell plasmalemma (2, 44, 47, 57). In these species, contact between parasite and host cell occurs

at the anterior tip of the parasite. As parasite entry progresses, invagination of the host cell plasmalemma enlarges to form a parasitophorous vacuole around the parasite (2). At no time does the host plasmalemma become discontinuous. This process has been likened to induced phagocytosis (2). However, it is generally accepted that apicomplexans actively penetrate rather than being phagocytosed by host cells (2, 45, 74, 85). In contrast, other studies involving Eimeria spp. have shown that the host cell plasmalemma is disrupted at the site of parasite entry and a separate parasitophorous vacuole membrane is formed around the parasite within the host cell cytoplasm (85, 111, 114). The apparent difference in E. magna penetration as compared to other Eimeria spp. may reside in the fact that E. magna penetrates at a relatively slower rate than other Eimeria spp. (Dr. C.A. Speer, personal communication). Additional studies have shown that zoites of Toxoplasma gondii may also disrupt the host cell plasmalemma during penetration (74, 75).

Sporozoites and merozoites of several Eimeria spp. have been found to penetrate and exit several cells in vitro before finally remaining intracellular and undergoing further development. As zoites leave cells, the host cell cytoplasm may escape at the site of exit but seldom does host cell cytoplasm escape during parasite penetration (85). Parasites leaving cells often carry a thin layer of host

cell plasmalemma and cytoplasm with them (85). Whether this host cell material functions in antigen-masking or some other evasion of host defense mechanisms is not known.

In vitro cultivation of various intracellular coccidian parasites such as Toxoplasma gondii, Besnoitia spp., Isospora spp., and Sarcocystis spp. as well as Eimeria spp. has been described (110). These parasites have the ability to penetrate and develop in several different cell lines, yet they appear to develop best in cell lines derived from their natural hosts (110). Sporozoites of several Eimeria spp. which infect avian or mammalian hosts will develop in vitro to mature or immature second-, third- or fourth-generation meronts; however, only E. tenella has been grown from sporozoites to oocysts in cell culture (20, 110). Other studies have shown that merozoites from certain species of Eimeria, including E. bovis, which are obtained from infected hosts can develop to advanced endogenous stages in cell culture (110). For example, Speer and Hammond (112) demonstrated that first-generation E. bovis merozoites taken from calves 14 days after inoculation of oocysts, would develop to mature second-generation meronts, gamonts and oocysts in primary embryonic bovine kidney cell cultures. Alternatively, sporozoites of E. bovis will develop only to mature first-generation meronts in cell cultures (28, 84). This evidence implies that certain factors or conditions which are necessary for complete

endogenous development of most <u>Eimeria</u> spp. (except <u>E.</u> <u>tenella</u>) are lacking in present day cell culture systems (110).

Immunity

Immunity to coccidiosis refers to the reduction or disappearance of clinical signs or oocyst passage upon subsequent challenge of the host with oocysts of the same species that elicited a primary infection. Most species of <u>Eimeria</u> are immunogenic and cause some degree of resistance to reinfection. However, resistance is generally specific with little cross-reactivity between parasite species (8, 86, 94, 97).

Both humoral and cell-mediated mechanisms have been implicated in acquired immunity to coccidiosis, but their respective roles have not been well defined (37, 72, 90, 91, 94, 95, 102). Immunofluorescent antibody and precipitation assays have shown that a systemic IgG response occurs in the host during infections (50, 65, 86, 120). For example, Andersen et al. (1) using immunofluorescent antibody techniques, demonstrated that a specific IgG response is first detectable against first-generation merozoites of <u>E.</u> <u>bovis</u> about 14 days after per os inoculation of calves with a million oocysts. This IgG response reached its peak 7 days later and was still detectable at 98 days after inoculation (1).

Parasite-specific IgG has been shown to cause in vitro complement-mediated lysis as well as immobilization and opsonization of Eimeria sporozoites and merozoites (1, 90, 91, 103). However, IgG antibodies are not likely to contribute to protection against Eimeria spp. because they are not normally found in body secretions (19). However, increased vascular permeability resulting from inflammatory processes that are elicited by the disease may allow interaction between IgG and the parasite (103).

Existing evidence indicates that maternal transfer of immunity affords some degree of protection against Eimeria infections in poultry (89). Passive transfer of immune serum in chickens and rats has been shown to decrease the severity of infections (88, 93, 95). For example, Rose (88) demonstrated that daily administration of immune serum by intraperitoneal injections in conjunction with intravenous injections resulted in as much as 50% decrease of E. maxima oocyst production in susceptible young chickens. However, the degree of protection varied directly with the volume of immune serum injected and inversely with the size of oocyst challenge inoculum (88). There have been no reports on the effects of passive transfer of immune serum on bovine coccidiosis.

IgA immunoglobulins may be involved in the immune response to coccidia because of their secretory nature and ability to withstand exposure to proteolytic enzymes (7, 16,

18, 19, 21). In general, it is thought that the secretory immune system functions by immobilizing microorganisms or antigens on mucosal surfaces, thus impeding their entry into host tissues (15, 94, 102). There have been no reports concerning IgA involvement with E. bovis infections in cattle. Davis et al. (16) reported that the concentration of secretory IgA in the intestinal lumen of chickens increased dramatically following infections with E. tenella. Additionally, Douglass and Speer (21) described the adherence of enterocyte-associated mucus or intestinal contents of immune mice to E. falciformis sporozoites. Immune enterocyte-associated mucus or intestinal contents also caused agglutination of sporozoites as well as significantly shorter length/width ratios than sporozoites exposed to normal enterocyte-associated mucus (21). They attributed the adherence of immune material, sporozoite agglutination and difference in length/width ratios to the parasite-specific secretory IgA present in the enterocyte-associated mucus and intestinal contents of immune animals (21).

Little or no information exists on the role of IgM or IgE in immunity to Eimeria species. Rose et al. (100) reported that a specific IgM response was rapid and of short duration, about 20 days, in rats which were exposed to E. nieschulzi oocysts. No apparent anamnestic IgM response resulted from a challenge inoculation of E. nieschulzi oocysts (100).

Several authors have reported on the ability of various protozoan parasites such as _Trypanosoma_ spp., _Leishmania_ spp., _Toxoplasma gondii_ and _Eimeria_ spp. to redistribute (cap formation) and shed immune complexes from their surfaces (5, 11, 12, 22-25, 117, 126). Capping may represent a mechanism by which parasites evade host humoral responses. However, the presence of fixed antigenic sites of _T. gondii_ and _E. nieschulzi_ demonstrate that capping and subsequent shedding of immune complexes on parasite surfaces during cell penetration is probably not complete (11, 23).

Although parasite-specific immunoglobulins may modulate or play a direct role in _Eimeria_ infections, experiments with T- and B-cell deficient animals imply that cell-mediated immunity (CMI) may be more important than immuno-globulins. Rose and Hesketh (95) found that approximately 3 times as many _E. nieschulzi_ oocysts were passed in homozygous nu/nu (athymic) rats as compared to heterozygous nu/+ (euthymic) rats, and in contrast to nu/+ rats, nu/nu rats were completely susceptible to reinfection. Additionally, nu/nu rats were unable to produce agglutinating antibodies directed against sporozoites, whereas serum transferred from immune nu/+ rats to nu/nu or nu/+ rats afforded a reduction in oocyst production in both nu/nu and nu/+ animals during primary infections (95). Conversely, bursectomized chickens, although slightly more susceptible than controls to challenge inoculations of _E._

maxima oocysts, were substantially immune (95). These results imply that T-lymphocytes are essential for immunity and their major effect is exerted in some fashion other than acting merely as helper T-cells for immunoglobulin production. Recently, Rose and several others have described a rapid depletion of circulating T-cells in immune animals upon challenge with Eimeria spp., followed by an increase in peripheral blood leukocytes with subsequent localization of these cells in the intestines of infected animals (101, 102, 104). Since this response was specific (101, 104) and a deficiency of T-cells causes the inability of animals to resist reinfection with their respective Eimeria spp. (72, 95, 96), this evidence is indicative of a functional T-cell response to coccidiosis (104).

Other investigators have demonstrated evidence of CMI responses in cattle following infections with E. bovis. Delayed hypersensitivity (DH) as well as lymphocyte blastogenesis was initiated by antigens derived from E. bovis oocysts (55). Additionally, a dialysable transfer factor (TF) prepared from the lymph nodes of immune calves was shown to render nonimmune animals partially immune to E. bovis (55). This effect was apparently species-specific since passive transfer of bovine TF did not protect rabbits or mice from coccidiosis, even though a cross-reacting DH response to E. stiedai or E. ferrisi, was detectable in recipient animals (54, 55). Speer et al. (113) have

recently shown that a lymphokine(s) from concanavalin A-stimulated bovine T-cells, significantly inhibited the intracellular development of E. bovis sporozoites to merozoites in an established bovine monocyte cell line (M617), whereas most E. papillata (murine) sporozoites were destroyed intracellularly in the same lymphokine-treated cell line. This evidence underscores the ability of certain specific T-cell products to stimulate nonspecific effects in their target cells.

The portion of the eimerian life cycle which is most affected by host immune responses has not been precisely determined. Evidence concerning several species of eimerians implies that resistance to reinfection is directed mainly against the asexual invasive stages, namely sporozoites and merozoites (38). However, several investigators reported no significant difference in the numbers of intracellular invasive stages of the parasite in immune hosts as compared to nonimmune hosts (38). In the case of E. bovis infections in cattle, Hammond et al. (39) suggested that the immune response chiefly affects the gametogenous stages of the parasite. From this information, it appears that different species of Eimeria are probably affected in different ways by their respective host's immune responses. This is probably a reflection on the many different sites of infection and subsequent development by species-specific parasites in numerous host species.

Monoclonal Antibodies

Monoclonal antibodies (MAbs) are defined as identical copies of antibody containing 1 heavy chain class and 1 light chain type (32). The advent of somatic cell hybridization techniques, fusing activated lymphocytes with plasmacytomas in order to create continuous cell lines (hybridomas) that secrete almost unlimited quantities of MAbs with a predefined specificity, has revolutionized the field of immunology. MAb preparations are virtually free of the nonspecificity and cross-reactivity consequences that are encountered with conventional antisera. Additionally, since MAbs are immunologically homogeneous, there is no need to ensure specificity by tedious cross-absorption experiments which are necessary for the production of monospecific antisera. For these reasons, MAbs have become valuble immunochemical reagents.

In a brief historical outline of hybridoma technology, Goldsby et al. (33) stated that in 1973 Schwaber and Cohen (108) produced the first antibody-secreting hybridomas by using Sendai virus to fuse human lymphocytes to a mouse plasmacytoma (myeloma). This report was the first to establish the feasibility of fusing mouse myeloma with lymphocytes of another species in order to produce nonmurine MAbs. However, it was Kohler and Milstein (56) who in 1975 developed a rational and selective strategy for the

construction of hybridomas that secrete MAbs of desired
specificity.   In order to accomplish this, Kohler and
Milstein capitalized on the earlier work of Littlefield (63)
who in 1964 selected somatic cell hybrids on the basis of
the HAT (hypoxanthine, aminopterin, and thymidine) system in
conjunction with mutant myeloma cell lines that were lacking
in 1 or both hypoxanthine guanine phosphoribosyl
transferase (HGPRTase) and thymidine kinase salvage enzymes.
Since aminopterin blocks the enzymes necessary for the "de
novo" synthesis of DNA, and the myeloma cells are deficient
in HGPRTase or thymidine kinase, only hybrids between the
myeloma and normal cells will grow when placed in HAT medium
(41).   Myeloma cells provide the immortality whereas normal
cells provide the salvage pathway enzymes that are necessary
for the incorporation of hypoxanthine and thymidine into DNA
synthesis (41). Taking this work 1 step further, Kohler
and Milstein (56) using inactivated Sendai virus, fused a
HAT-selectable mouse myeloma cell line (P3-X63-Ag8) with
spleen cells from mice which had been previously immunized
with sheep red blood cells.   After selection, they screened
the resulting hybridomas for the production of antibody
specific for sheep red blood cells.   Positive hybridomas
were subsequently cloned to initiate a monoclonal antibody-
secreting cell line from a single hybrid cell (56).
According to Goldsby et al. (33), the fusion procedures of
Kohler and Milstein were further simplified after Pontecorvo

in 1976, demonstrated that polyethylene glycol (PEG) solutions were able to mediate animal cell fusions in the place of inactivated Sendai virus. PEG is inexpensive and has made it possible to fuse cells in which 1 or both partners lack receptors for Sendai virus (33). Use of PEG has also allowed for the fusion of cells which are phylogenetically distinct (i.e. murine and bovine) (33). However, several of the HAT-sensitive myeloma cell lines that were available at that time expressed immunoglobulin heavy and light chains. Thus, many of the resulting hybridomas produced both myeloma and antigen-specific immunoglobulins (56). In 1979, Kearney et al. (53) overcame this problem by isolating a variant (PX-X63-Ag8.653) of the PX-X63-Ag8 myeloma fusion partner which had lost the ability to express immunoglobulin but still permitted the construction of antigen-specific antibody-secreting hybrids. Other myeloma fusion partners which do not secrete immunoglobulins are now available as well (41). Presently, procedures which incorporate the aforementioned modifications of the basic techniques established by Kohler and Milstein are considered state of the art in monoclonal antibody technology.

Danforth (14) described the development of monoclonal antibodies directed against E. tenella and E. mitis sporozoites. At least 8 different binding patterns on or in sporozoites were determined by indirect fluorescence

antibody tests (14). Speer et al. (115, 116) using indirect immunocytochemical techniques in conjunction with transmission and scanning electron microscopy, demonstrated the ultrastructural localization of monoclonal IgG antibodies against E. tenella oocysts, sporocysts and sporozoites and their effects on E. tenella sporozoites. Oocysts, sporocysts and sporozoites of E. tenella were found to possess common antigens (116). Monoclonal IgG antibodies also caused complement-mediated lysis of E. tenella sporozoites, altered surface texture, and significant shortening of sporozoites (115). More recently, anti-sporozoite surface-reacting MAbs that inhibit sporozoite penetration of poultry-derived primary cell cultures have been reported for E. tenella and E. adenoides (3). Previous to the present investigation, there are no studies concerning the production of MAbs against stages of E. bovis.

MAbs have been used for the detection, isolation and purification of protective antigens of protozoan parasites such as Plasmodium yoelii and Babesia bovis (40, 127). Since these parasites are closely related to Eimeria spp., it is likely that Eimeria spp. possess similar protective antigens. However, there have been no published reports on the use of MAbs to isolate eimerian proteins. Since most studies have used crude parasite preparations consisting of oocysts, sporozoites and merozoites, there has been little

information on the roles of isolated antigens in humoral or CMI responses to Eimeria species.

Rationale

Since E. bovis sporozoites and merozoites exist for brief periods outside of host cells and have been shown to possess immunogenic proteins (82, 83), they may be susceptible to the actions of parasite-specific immunoglobulins. However, only the sporozoite stage will easily undergo intracellular development in continuous cell cultures. Therefore, parasite-specific immune serum (IS) as well as MAbs will be produced against E. bovis sporozoites and assessed for their ability to decrease sporozoite in vitro cell penetration and development. Comparison studies between the effects of IS and MAbs on sporozoite penetration and intracellular development should determine if unique parasite proteins exist which are crucial for these processes. For example, if exposure of sporozoites to MAbs decreases the ability of sporozoites to undergo cell penetration or development, then the parasite proteins that the MAbs are directed against will be identified by gel electrophoresis in conjunction with western blotting and compared to similar electrophoretically separated sporozoite antigenic profiles that are detected by IS. In this manner, the identity as well as immunogenicity of crucial MAb-identified parasite proteins will be established. On the

other hand, if only IS has a detrimental effect, then the immunogenic sporozoite proteins, especially those located on sporozoite surfaces, may be crucial for the process of sporozoite penetration or development. Radioiodination of sporozoites by lactoperoxidase followed by autoradiography and comparison to western blots that are probed with IS will indicate the presence of immunogenic sporozoite surface proteins.

Immunoelectron microscopy will be used to determine the ultrastructural localization of antibody receptors and the possible redistribution of immune complexes on the surfaces of E. bovis sporozoites. Additionally, immunocytochemical assays with parasite-specific MAbs in conjunction with light microscopy should resolve the fate of sporozoite antigens during the course of intracellular development as well. Because sporozoites penetrate host cells by their anterior end, careful ultrastructural observations will also allow for the determination of any structural changes in the parasite plasmalemma or apical complex as well as the host cell plasmalemma during and immediately after penetration.

The information obtained from these studies will increase our understanding of the effects of parasite-specific immunoglobulins on the crucial event of host cell penetration and intracellular development by E. bovis sporozoites and may indicate the presence of protective surface antigens (if any) of E. bovis sporozoites.

Monoclonal antibodies have been used to identify the protective antigens of <u>Plasmodium</u> spp. sporozoites (13).

Objectives

The objectives of this investigation are to produce parasite-specific IS and MAbs, and to determine the ultrastructural localization, possible redistribution of immune complexes on parasite surfaces and effects of these immunoglobulins on the process of in vitro host cell penetration and intracellular development by <u>E</u>. <u>bovis</u> sporozoites. The relative molecular weights, immunogenicity and surface localization of several sporozoite proteins will be established, and the fate of sporozoite surface antigens during the course of in vitro intracellular development will be determined. Any ultrastructural changes in the plasmalemmae of sporozoites or their apical complexes as well as host cell plasmalemmae during and after parasite penetration will be described as well.

MATERIALS AND METHODS

Experimental Animals

Holstein-Friesian bull calves from (1 to 7 days of age) were purchased at the Bozeman Livestock Auction. These animals were confined to units within the Marsh Laboratory Isolation Building for 3 to 4 weeks prior to inoculation with E. bovis. Just before experimentation, the animals were moved to the Marsh Laboratory Clinic, where they were inoculated with oocysts of E. bovis and kept throughout the course of infection or duration of experimental procedures. Surviving, healthy animals were resold at the Bozeman Livestock Auction. Milk replacer, hay, oats, clean straw for bedding and water were supplied to these animals as necessary. Isolation units or clinic stalls were thoroughly cleaned before occupation by newly received animals as well as twice a week during animal occupation.

Inbred BALB/cByJ mice that were used for the production of parasite-specific monoclonal antibodies were purchased and housed at the Animal Resource Center located on the MSU campus. Animal Resource Center personnel were responsible for the maintenance and care of these animals.

Continuous Cell Cultures

Madin-Darby bovine kidney (MDBK) cells (American Type
Culture Collection, Rockville, MD) and bovine monocytes
(M617) were used as in vitro host cells for E. bovis
parasites. The M617 cell line was obtained from blood
monocytes of a 6-year old Guernsey cow and kindly provided
to the Electron Microscopy laboratory by Dr. G.A. Splitter
(Department of Veterinary Science, University of Wisconsin-
Madison, Madison, WI 53706). The MDBK cells were
maintained in culture medium (CM) that consisted of RPMI
1640 (Gibco, Long Island, NY) plus 10% fetal bovine serum
(FBS, Hyclone Laboratories, Inc., Logan, UT), 2 mM L-
glutamine, 50 U of penicillin G per ml and 50 ug (microgram)
dihydrostreptomycin per ml. Similar CM was used for the
maintenance of M617 cells except that the concentration of
FBS was increased to 15% and 5×10^{-2} mM 2-mercaptoethanol was
added to each ml of CM.

The P3-X63-Ag8.653 mouse myeloma cell line that was
originally described by Kearney et al. (53) was purchased
from the American Type Culture Collection and used as the
fusion partner in the construction of hybridomas. Myeloma
cells were maintained in Dulbecco's Modified Eagle Medium
(DMEM; Gibco Laboratories, Chagrin Falls, OH) with 15% FBS
and similar concentrations of L-glutamine, penicillin G and
dihydrostreptomycin as described above. All serum

supplements were heat-inactivated at 56°C for 30 min before addition to the various CM and all cell cultures were incubated at 38°C in 5% CO_2-95% air.

Parasite

The strain of E. bovis used throughout the course of experimentation was obtained from Dr. Paul Fitzgerald (University of Illinois, Urbana, IL 61801). This strain of E. bovis was originally isolated in the state of Utah by Dr. D.M. Hammond (Dr. C.A. Speer, personal communication). The parasite was maintained by serial passage in outbred Holstein-Friesian bull calves.

Production, Collection and Storage of Oocysts

Approximately 18 days after an oral inoculation of 3.5×10^4 to 5×10^4 sporulated oocysts of E. bovis, infected calves (usually 2 calves at a time) were placed in separate elevated metal fecal collection stalls in which they were unable to turn around but could stand or lie down. Feces containing unsporulated oocysts of E. bovis, passed through expanded metal grates that were situated in the stall floors immediately beneath the hindquarters of the infected calves, and were collected in metal basins. Infected calves remained in the stalls for a period of 5 days and the feces were removed from the basins for further processing on a daily basis. Oocysts of E. bovis were separated from the

feces by sugar flotation, concentrated by centrifugation, and sporulated in aerated aqueous 2.5% (w/v) $K_2Cr_2O_7$ by the methods described by Davis (17). Sporulated oocysts were then pooled and stored at $4^{\circ}C$ in 2.5% $K_2Cr_2O_7$. Oocyst preparations were estimated to consist of at least 90% E. bovis and 10% or less of other bovine eimerian species by duplicate hemacytometer counts.

Sporozoite Isolation

Sporulated oocysts were treated with a 5.25% (w/v) aqueous sodium hypochlorite solution (Clorox) for 1 hr at room temperature (RT), and then centrifuged (200 xg(gravity)/10 min). Oocysts in the supernatant were decanted, diluted 1/2 with sterile calcium and magnesium deficient Hanks' balanced salt solution (HBSS, pH 7.4; Gibco, Santa Clara, CA) and centrifuged once again. The pellet of sporulated oocysts was then subjected to several additional washes with sterile HBSS to ensure removal of the sodium hypochlorite.

Clean sporulated oocysts were resuspended in HBSS and broken by grinding with a motor-driven Teflon-coated tissue grinder. When most of the sporocysts were released from the oocysts, the suspension containing fractured oocyst walls, sporocysts and rare intact oocysts was pelleted by centrifugation (200 xg/10 min), washed with HBSS and treated with excysting fluid (0.25% (w/v) trypsin 1/250, Gibco, Long

Island, NY; 0.75% (w/v) sodium taurocholate, Difco, Detroit, MI; in HBSS, pH 7.4) for 3 hr in a 38°C water bath to enable sporozoites to excyst from sporocysts. Following incubation, the parasite suspension was washed once with HBSS, resuspended in HBSS, and applied to a nylon wool (Leuco-Pak, Fenwal Laboratories, Deerfield, IL) column in order to remove sporocysts, oocyst walls and oocysts (60). The column eluate contained highly purified viable sporozoites and a few sporocysts, oocyst walls and oocysts.

Merozoite Isolation

The in vitro cultivation and isolation of E. bovis first-generation merozoites was accomplished as previously described by Reduker and Speer (82). Briefly, monolayers of M617 cells in 150 cm^2 polystyrene tissue culture flasks (Corning Glass Works, Corning, NY) were inoculated with 40 ml of fresh CM (2% FBS) containing 1.5x10^6 E. bovis sporozoites, and incubated at 38°C until mature meronts and extracellular merozoites were detected by phase-contrast microscopy. Merozoites were harvested from the flasks daily from days 10 to 21 after sporozoite inoculation. Each flask was gently rapped on the palm of the hand, rocked back and forth 20 times, then decanted into sterile 50 ml conical centrifuge tubes. Ten ml of HBSS was added to each flask and the process was repeated, followed by the addition of 40 ml of fresh CM (2% FBS) to each flask which was then

returned to the incubator. The harvested suspensions which contained merozoites and some host cells, were pelleted by centrifugation, resuspended in 2 to 3 ml of HBSS and disturbed by 8 to 10 strokes with a Teflon-coated motor-driven tissue grinder in order to disrupt any intact mature meronts. The suspensions were pooled and subjected to purification by nylon wool columns (60, 81). This procedure generated highly purified suspensions of first-generation merozoites.

Normal Serum

Several noninfected 2 to 3 week old calves were bled by venipuncture with sterile 20 ml evacuated blood collection tubes (Becton Dickinson and Company, Rutherford, NJ) and 18 gauge, 1 1/2 inch sterile hypodermic needles (Becton Dickinson and Company, Rutherford, NJ). The blood was allowed to clot on ice and then centrifuged at 1500 xg for 10 min, after which the serum was removed, heat-inactivated at 56°C for 30 min in a water bath, aliquoted into 1 ml samples and stored at -20°C. Henceforth, this serum pool is referred to as normal serum (NS) and was used for various negative antibody controls.

Serum for Parasite-specific IgG Titrations

Two calves that had never been exposed to E. bovis were bled by venipuncture and then inoculated orally with 3.5×10^4

sporulated E. bovis oocysts. Beginning with the day of inoculation, blood samples (10 ml) were drawn 3 times a week from each calf for 6 weeks. After the clinical signs and passage of unsporulated oocysts due to the primary infection had disappeared, the calves were subjected to an oral challenge inoculum of 5×10^4 oocysts and bled 3 times a week for 8 weeks. The relative numbers of unsporulated oocysts per gram of feces passed during the challenge infection were determined for each calf.

The serum was removed from all blood samples by a process similar to that described above for the normal serum samples. One ml aliquots of serum from the individual blood samples were removed, labelled and stored at $-20^\circ C$ until the respective IgG titers could be determined by immunofluorescence assays.

Antiserum

A calf that was initially free of E. bovis infection, received an intravenous (IV) inoculation of 2×10^7 E. bovis sporozoites followed by an IV challenge with 2×10^7 sporozoites 6 weeks later. One week after challenge, 50 ml of venous blood were collected, from which the serum was removed, heat-inactivated, aliquoted into 1 ml samples and stored at $-20^\circ C$. This serum pool is referred to as antiserum (AS).

Immune Serum

A calf that had previously survived an experimental infection induced by oral inoculation of 4×10^4 sporulated oocysts of E. bovis, was challenged 5 weeks after the primary dose with a similar oral dose of oocysts. At 4 and 8 weeks after the initial challenge, the calf was inoculated orally with 5×10^4 and 1×10^5 oocysts, respectively. One week after the final challenge, immune serum (IS) was collected as described above, heat-inactivated, aliquoted and frozen at $-20^{\circ}C$.

Conjugates

Fluorescein-conjugated goat antimouse IgG (heavy and light chain specific), rabbit antibovine IgG (heavy and light chain specific; United States Biochemical Corporation, Cleveland, OH) and ferritin-conjugated rabbit antimouse (United States Biochemical Corporation, Cleveland, OH) or antibovine IgG (heavy and light chain specific; E Y Laboratories, San Mateo, CA) were used to detect parasite-specific antibodies by light (LM) and transmission electron microscopy (TEM), respectively. Horseradish peroxidase-conjugated rabbit antimouse IgG (heavy and light chain specific; United States Biochemical Corporation, Cleveland, OH) as well as horseradish peroxidase-conjugated rabbit antibovine IgG (heavy and light chain specific; Cappel

Laboratories, Cochranville, PA) were used to detect parasite-specific antibodies in conjunction with enzyme-linked immunosorbent assays or western blotting techniques. All conjugated immunoglobulins were handled, reconstituted or diluted according to the specifications of the manufacturer.

Indirect Immunofluorescence Assays

The indirect fluorescent antibody (IFA) technique used here was similar to the procedure described by Burgess et al. (10). Concentrated suspensions (0.02 ml) of purified sporozoites or merozoites (approximately 1,500 organisms per well) of E. bovis were placed on multi-welled toxoplasmosis microscope slides (Bellco Glass, Inc., Vineland, NJ), air-dried, fixed in acetone (prefixed) and stored at -20°C in plastic slide boxes until used. Sera from immunized or non-immunized animals, hybridoma ascites or CM were appropriately diluted with phosphate buffered saline (PBS, 0.15 M, pH 7.4), applied to multi-welled toxoplasmosis slides containing sporozoites or merozoites of E. bovis and incubated for 45 min at RT in high humidity. Specimens on slides were then washed in PBS, incubated with fluorescein-conjugated antiglobulins (diluted 1/10 with 0.05 M PBS) for 30 min at RT, washed twice in PBS, once in distilled water and air-dried. Three drops of mounting fluid (60% (v/v) glycerol in PBS) were added to each slide followed by

application of a glass coverslip. The slides were then examined by phase-contrast and epifluorescence with a Nikon Labophot light microscope. These IFA assays were used to titer the serum of immunized animals, to screen hybridoma supernatants as well as to determine the appropriate titers of AS, IS or MAbs to use in parasite inhibition assays, immunoelectron microscopy or detection of antigens on western blots. Parasite-specific antibody titers will be reported herein as the reciprocal of the highest dilution in which a positive IFA result was obtained.

The ability of specific MAbs to react with parasite surface antigens were determined by modified IFA procedures as follows (called live IFA) (42). Approximately $3x10^6$ live sporozoites or merozoites were reacted with 0.5 ml of heat-inactivated ascites or CM of each MAb-secreting clone in microfuge tubes (Sarstedt, Inc., St. Louis, MO) for 45 min at RT. The specimens were then washed in HBSS, fixed with 0.2% (v/v) glutaraldehyde in Millonig's phosphate buffer (postfixed) for an additional 30 min at RT, washed in HBSS, incubated with fluorescein-conjugated goat antimouse IgG for 30 min, washed twice in HBSS, applied to microscope slides, covered by glass coverslips and examined by fluorescence microscopy.

Enzyme-linked Immunosorbent Assays

The enzyme-linked immunosorbent assay (ELISA) method used herein was similar to established procedures with minor modifications (59, 124). Briefly, 96-well Immulon 1 plates (Dynatech Laboratories, Alexandria, VA) were coated with 5×10^4 purified sporozoites of E. bovis per well in 50 ul (microliter) of 0.05 M bicarbonate buffer (0.43% (w/v) Na_2CO_3; 0.3% (w/v) $NaHCO_3$; 0.2% (w/v) sodium azide in distilled water; pH 9.6). The plates were incubated overnight at 4^OC with the lids in place. Subsequently, the plates were decanted, washed 3 times in Tween phosphate buffered saline (TPBS, 0.05% (v/v) Tween 20 in PBS, pH 7.4), washed once in distilled water, dried completely and stored at -20^OC under airtight conditions. During the course of the assay, plates were allowed to equilibrate to RT, 100 ul of hybridoma supernatants or negative controls were added to selected wells and the plates were incubated for 1 hr at RT in high humidity. Each well was then washed 3 times in TPBS, incubated with 50 ul of horseradish peroxidase-conjugated anti-immunoglobulin (diluted 1:400 in TPBS) for 1 hr and washed 3 times with PBS (without Tween 20). After the final wash, 50 ul of substrate (0.2 mg/ml solution of 2, 2´-azinobis (3-ethylbenzthiazoline sulfonic acid); Sigma, St. Louis, MO) in citrate phosphate buffer (0.15 M, pH 5.3) and 50 ul of 0.03% (v/v) H_2O_2 in distilled water were added

to each well (124). The plates were read visually after a 30 min incubation time at RT. The above procedures were mainly used in screening primary as well as cloned hybridoma supernatants for the presence of antibodies. However, MAbs were assigned immunoglobulin classes and subclasses by similar methods using a commercial ELISA mouse monoclonal isotyping kit (Hyclone Laboratories, Logan, Ut).

Monoclonal Antibody Production

Seven parasite-specific MAbs were kindly provided by the Immunology Laboratory of the Department of Veterinary Science and further characterized in the Electron Microscopy (EM) Laboratory. Other parasite-specific MAbs were generated in the EM Laboratory by the following procedures. Adult female BALB/cByJ mice were immunized by intraperitoneal inoculation (IP) of approximately 4×10^6 purified E. bovis sporozoites that had been previously emulsified 1:1 in Freund's complete adjuvant (Difco Laboratories, Detroit, MI) and HBSS. The parasite-specific antibody titer of each immunized mouse was determined by IFA over the course of approximately 1 month. When antibody titers decreased to low or negative levels, mice were boosted IP with a similar dose of live sporozoites in 0.5 ml HBSS. Three days after the booster inoculation, spleens from the immunized mice (usually 2 per fusion) were aseptically removed and teased apart in HBSS to free

individual splenocytes from the connective tissue of the spleens. The splenocytes were then suspended in a solution containing 1 ml HBSS and 9 ml sterile triple distilled water for 5 sec in order to lyse residual erythrocytes. After the addition of 1 ml of sterile 10X concentrated saline (8.5% (w/v) NaCl in distilled water) to re-establish isotonicity, the remaining intact splenocytes were washed free of erythrocyte membrane debris, resuspended in 10 ml HBSS and counted with a hemacytometer. After enumeration, 10^8 splenocytes were copelleted with 5×10^7 logarithmically growing P3-X63-Ag8.653 mouse myeloma cells and fused in a 50% (w/v) polyethylene glycol solution in HBSS according to the methods of Galfre et al. (31). The cells were distributed into the wells of 24-well tissue culture plates (Corning Glass Works, Corning, NY) that had been previously seeded with BALB/cByJ mouse thymocytes (2×10^5 cells per well) and grown in selective DMEM containing 100 uM hypoxanthine, 0.4 uM aminopterin and 16 uM thymidine (HAT medium; Sigma, St. Louis, MO) and 15% heat-inactivated FBS or horse serum (HS; Hyclone Laboratories, Logan, UT). The resulting hybridoma cultures were maintained for 10 weeks with 3 changes of HAT medium over the first 10 days followed by biweekly changes of DMEM with hypoxanthine and thymidine but without aminopterin (HT medium). Once the hybridoma cultures reached confluency, sporozoite-specific antibody secreting hybrids were detected by IFA or ELISA techniques.