



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 immunologically important.

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

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## ABSTRACT

The 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: EUCCOCCIDIORIDA 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 worldwide 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 coccidiosis 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  $\mu\text{m}$  (micrometer) by 200  $\mu\text{m}$  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 development to second-generation meronts (36). Second-generation meronts are relatively small (about 10  $\mu\text{m}$  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 Eimeria spp. (except E. tenella) 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 Eimeria 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 E. bovis 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 immunoglobulins. 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 valuable 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.

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Monoclonal antibodies have been used to identify the protective antigens of Plasmodium 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 E. bovis 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  $5 \times 10^{-2}$  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<sub>2</sub>-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  $3.5 \times 10^4$  to  $5 \times 10^4$  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°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<sup>2</sup> polystyrene tissue culture flasks (Corning Glass Works, Corning, NY) were inoculated with 40 ml of fresh CM (2% FBS) containing  $1.5 \times 10^6$  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  $3.5 \times 10^4$

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  $5 \times 10^4$  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  $-20^{\circ}\text{C}$  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  $2 \times 10^7$  E. bovis sporozoites followed by an IV challenge with  $2 \times 10^7$  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  $-20^{\circ}\text{C}$ . 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  $4 \times 10^4$  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  $5 \times 10^4$  and  $1 \times 10^5$  oocysts, respectively. One week after the final challenge, immune serum (IS) was collected as described above, heat-inactivated, aliquoted and frozen at  $-20^{\circ}\text{C}$ .

### 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  $3 \times 10^6$  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  $5 \times 10^4$  purified sporozoites of E. bovis per well in 50  $\mu$ l (microliter) of 0.05 M bicarbonate buffer (0.43% (w/v)  $\text{Na}_2\text{CO}_3$ ; 0.3% (w/v)  $\text{NaHCO}_3$ ; 0.2% (w/v) sodium azide in distilled water; pH 9.6). The plates were incubated overnight at 4°C 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°C under airtight conditions. During the course of the assay, plates were allowed to equilibrate to RT; 100  $\mu$ l 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  $\mu$ l 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  $\mu$ l 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  $\mu$ l of 0.03% (v/v)  $\text{H}_2\text{O}_2$  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  $4 \times 10^6$  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  $5 \times 10^7$  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 ( $2 \times 10^5$  cells per well) and grown in selective DMEM containing 100  $\mu$ M hypoxanthine, 0.4  $\mu$ M aminopterin and 16  $\mu$ M 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.

Positive hybrids were cloned by limiting dilution in 96-well microtiter plates (Corning Glass Works, Corning, NY) and screened by IFA or ELISA (41). The resulting clones which secreted sporozoite-specific MAbs were then expanded in DMEM with 15% FBS or HS and subsequently frozen at  $-195^{\circ}\text{C}$  in liquid nitrogen until needed. CM from the cloned hybrids as well as heat-inactivated ascites from previously pristane (Sigma, St. Louis, MO) primed BALB/c mice inoculated with these cell lines, served as sources of parasite-specific MAbs. MAbs used for immunodetection of specific sporozoite antigens in conjunction with intracellular development, immunoelectron microscopy and western blots, were concentrated from CM by precipitation in saturated ammonium sulfate solution (pH 7.2) and dialyzed against distilled water. The concentrated MAbs were then dissolved in 0.15 M PBS (pH 7.4) and stored at  $-70^{\circ}\text{C}$ .

#### Parasite Inhibition Assays

The MDBK cells that were used for these assays were adapted to DMEM from RPMI 1640 CM. Following adaptation, confluent monolayers of MDBK cells were removed from 75 cm<sup>2</sup> tissue culture flasks (Corning Glass Works, Corning, NY) by trypsinization, washed in HBSS and resuspended in DMEM plus 15% HS. The average number of cells was determined by duplicate hemacytometer counts and each chamber of 8-chambered Lab-Tek tissue culture microscope slides (Miles

Scientific, Naperville, IL) was inoculated with 0.3 ml of DMEM (15% HS) containing  $3 \times 10^4$  MDBK cells. The cultures were incubated at 38°C in 5% CO<sub>2</sub>-95% air for 24 hr. Prior to the completion of this incubation interval, groups of freshly excysted and purified sporozoites of E. bovis were exposed to CM containing various MAbs with IFA titers of 10 or 20 as well as unfused mouse myeloma (Ag8) CM or several dilutions of AS, IS, with IFA titers of 160 and 80, respectively, and NS in PBS. All sporozoite groups were incubated in their respective immunoglobulin or control solutions for 30 min at RT, washed in HBSS and resuspended in DMEM plus 2% HS.

After the completion of the 24 hr incubation interval, the CM was removed from the MDBK cultures and 4 chambers of a slide were each inoculated with 0.3 ml DMEM (2% HS) containing  $1.5 \times 10^4$  sporozoites that had been previously treated with MAbs, AS, IS, NS or control solutions. The other 4 chambers were each inoculated with 0.3 ml DMEM (2% HS) containing  $1.5 \times 10^4$  untreated sporozoites. All cultures were then incubated as above for 24 hr, fixed in Bouin's fluid, stained in Giemsa's stain (1/20 in distilled water) and examined by bright field LM for intracellular sporozoites.

To determine the effect of parasite-specific immunoglobulins on intracellular development of sporozoites

of E. bovis, monolayers of MDBK or M617 cells in 8-chambered Lab-Tek microscope slides were inoculated with MAbs, Ag8 CM, AS, IS or NS treated sporozoites as described above, incubated at 38°C for 10 days, fixed in Bouin's, stained with Heidenhain's iron hematoxylin and examined by LM for meront development.

The following experiment was performed to determine if pretreatment of MDBK cells with CM containing MAbs would have an effect on the numbers of intracellular sporozoites. Monolayers of MDBK cells in 4 chambers of each of two 8-chambered Lab-Tek slides were exposed to CM containing MAbs with IFA titers of 20 for 30 min at RT, rinsed in HBSS and each well inoculated with  $1.5 \times 10^4$  untreated sporozoites in DMEM (2% HS). The remaining chambers of the 2 slides were exposed to DMEM (2% HS) for 30 min, rinsed in HBSS and inoculated with a similar number of untreated sporozoites in DMEM (2% HS). Following incubation at 38°C for 24 hr, the cultures were fixed, stained in Giemsa's stain and examined by LM.

Quantitative data were obtained by recording all of the intracellular sporozoites in each of 5 or 10 microscope fields of view or all of the intracellular meronts per chamber at X 400 magnification as one count. Four counts and a mean were then recorded for each experimental group. The data were statistically analyzed by Student's t test or

Tukey's (Studentized range) single factor analysis of variance (68).

#### Immunodetection of Antigens During Parasite Development

The chambers of 9 Lab-Tek tissue culture microscope slides were each seeded with  $3 \times 10^4$  MDBK cells in 0.3 ml DMEM (15% HS). Following a 24 hr incubation period at  $38^\circ\text{C}$  in 5%  $\text{CO}_2$ -95% air, the monolayers were inoculated with 0.3 ml DMEM (2% HS) containing  $1.5 \times 10^4$  purified sporozoites of E. bovis and incubated as above. At daily intervals for 8 days and then at 12 days after sporozoite inoculation, 1 slide was prefixed in acetone, stored at  $-20^\circ\text{C}$ , later allowed to equilibrate to RT, exposed to CM that contained sporozoite surface-reactive MAbs or Ag8 and processed for IFA as previously described.

#### Immunoelectron Microscopy

Approximately  $10^7$  (per sample) purified E. bovis sporozoites were prefixed with 0.15% (v/v) glutaraldehyde in Millonig's phosphate buffer (MPB; pH 7.4) for 30 min at RT in 15 ml polypropylene centrifuge tubes (Fisher Scientific, Kent, WA), washed twice with HBSS, centrifuged and reacted with AS, IS, NS, concentrated MAbs or Ag8 for 45 min at RT. The samples were then washed twice as before and then exposed to 0.5 ml ferritin-conjugated antimouse or antibovine immunoglobulins for 30 min at RT. After 2

additional washes in HBSS, the samples were centrifuged for 10 min at 200 xg and the pellets fixed with 2.5% (v/v) glutaraldehyde in MPB, postfixed in 1% (w/v)  $\text{OsO}_4$ , dehydrated in ethanol, and embedded in Spurr's medium. Thin sections were prepared with a Sorvall MT 5000 Ultra Microtome (DuPont, Wilmington, DE), stained with uranyl acetate and lead citrate, and examined by a JEOL 100 CX transmission electron microscope.

To determine the fate of immune complexes on sporozoite surfaces, live purified sporozoites were exposed to heat-inactivated AS, IS or concentrated sporozoite surface-reacting MAbs for 5, 10 or 20 min at RT, fixed with 0.15% (v/v) glutaraldehyde, washed twice with HBSS, exposed to appropriate ferritin-conjugated anti-immunoglobulins, washed twice in HBSS, fixed in 2.5% (v/v) glutaraldehyde and processed for TEM as described above.

#### Sporozoite Penetration of Cultured Cells

A suspension of MDBK cells ( $5 \times 10^6$ ) in 3 ml HBSS was inoculated with an equal volume of HBSS containing  $10^7$  purified live sporozoites, incubated for 5 or 10 min at RT, pelleted by centrifugation, fixed in 2.5% (v/v) glutaraldehyde in MPB overnight at  $4^\circ\text{C}$ , washed 3 times in 0.15 M cacodylate buffer (pH 7.4) and postfixed with a  $\text{OsO}_4$  and ruthenium red (Sigma, St. Louis, MO) solution for 3 hr at RT as described by Luft (67). Specimens were then washed in

cacodylate buffer, dehydrated in a graded series of ethanol, and embedded in Spurr's medium. Thin sections were stained with uranyl acetate and lead citrate and examined with a JEOL CX 100 electron microscope.

### Polyacrylamide Gel Electrophoresis

Purified sporozoites of E. bovis were solubilized in sodium dodecyl sulfate (Pierce Chemical Company, Rockford, IL) solubilizing solution (2% sodium dodecyl sulfate (SDS), 10% (v/v) glycerol,  $6.25 \times 10^{-2}$  M Tris-HCl (pH 6.8), with or without 4% (v/v) 2-mercaptoethanol) at  $100^{\circ}\text{C}$  for 15 min at a ratio of  $6 \times 10^6$  sporozoites to 10  $\mu\text{l}$  of solubilizing solution (83). The samples as well as prestained molecular weight standards (BRL; Bethesda Research Laboratory, Bethesda, MD) were subjected to polyacrylamide gel electrophoresis (SDS-PAGE) in 12.5% polyacrylamide slab gels using a discontinuous buffer system as described by Laemmli (58). Following electrophoresis (40 mA for approximately 3 hr), the gels were removed from the gel apparatus and fixed overnight in 25% (v/v) isopropyl alcohol with 7% (v/v) glacial acetic acid in distilled water. The resolved sporozoite proteins were either visualized by staining the gels with 0.25% (w/v) Coomassie Brilliant Blue (Sigma, St. Louis, MO) in the above fixer or subjected to western blotting techniques.

Approximate relative molecular weights ( $M_r$ ) were calculated from a calibration curve which was established by linear regression (34). Briefly, the logarithm of protein standard molecular weights plotted against their respective relative mobilities (distance of protein migration/distance of tracking dye migration) demonstrated a linear relationship (34). The approximate molecular weights of sporozoite proteins were estimated from this plot according to their relative mobilities.

#### Western Blotting

Sporozoite proteins were electrophoretically transferred (70 V for approximately 4 1/2 hr) from 12.5% SDS-polyacrylamide slab gels to nitrocellulose paper in a Bio-Rad Trans-Blot Cell (Bio-Rad, Richmond, CA) using a transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol) as described by Towbin et al. (121). Following transfer, the nitrocellulose sheets were fixed in 20% (v/v) methanol, 10% (v/v) acetic acid in distilled water for 15 min, washed twice in distilled water, air-dried and stored at  $-20^{\circ}\text{C}$  under air-tight conditions.

#### Immunodetection of Sporozoite Antigens on Nitrocellulose

Nitrocellulose sheets which supported resolved proteins of E. bovis sporozoites were incubated in bovine lacto transfer technique optimizer (BLOTTO) for 1 hr at RT to

block nonspecific binding sites (48). The nitrocellulose sheets were then cut into 4 mm wide strips, probed with AS, IS, NS, concentrated MAbs and Ag8 (diluted 1/20 in BLOTTO) in a moist chamber at 4°C overnight, washed 3 times (15 min each) in TPBS and incubated with a 1/200 dilution of horseradish peroxidase-conjugated anti-immunoglobulin in BLOTTO for 1 hr at RT. The strips were then washed twice in PBS, twice in 0.05 M Tris-0.2 M NaCl (pH 7.4), incubated for 1 hr at RT in a peroxidase substrate solution consisting of 0.3% (w/v) 4-chloro-1-naphthanol, 16.6% (v/v) methanol and 5 ul of 30% H<sub>2</sub>O<sub>2</sub> (Sigma, St. Louis, MO) in 0.05 M Tris-0.2 M NaCl (pH 7.4), washed twice in distilled water and air-dried (122). Molecular weights of immunodetected sporozoite antigens were calculated from prestained molecular weight standards which had been transferred to nitrocellulose from 12.5% SDS-polyacrylamide gels as described above.

#### Radioiodination and Autoradiography

Live purified sporozoites were surface-radioiodinated using lactoperoxidase by the procedures outlined by Reduker and Speer (83). Sporozoites were pelleted in a microfuge tube and resuspended in 50 ul of 0.15 M PBS (pH 7.4) containing 1 mg per ml lactoperoxidase (Sigma, St. Louis, MO) and 10 ul of a 10<sup>-5</sup> M solution of potassium iodide. Approximately 300 microcuries of carrier free <sup>125</sup>I-sodium iodide (New England Nuclear, Boston, MA) was added to this

suspension, followed by the addition of 5  $\mu$ l 0.6% (v/v)  $H_2O_2$  in PBS every 2.5 min for 10 min with mixing. At 12.5 min, 1 ml cold PBS containing 5 mM cysteine-HCl was added to stop the reaction and the mixture was centrifuged for 10 min at 250 xg. The radioiodinated sporozoites were washed twice in cold PBS-cysteine-HCl, once in cold PBS, centrifuged and solubilized at RT in SDS solubilizing solution without 2-mercaptoethanol. The radioiodinated samples were subjected to SDS-PAGE in a 12.5% polyacrylamide gel. The gel was fixed, dried with a slab gel dryer (Pharmacia, Piscataway, NJ) and exposed to Kodak X-OMAT AR X-ray film (Kodak, Rochester, NY) for 1 to 14 days at  $-70^{\circ}C$ . The exposed film was subsequently developed in Kodak GBX developer (Kodak, Rochester, NY) for 5 min, washed for 1 min in  $20^{\circ}C$  tap water, fixed in undiluted Kodak Rapid Fix (Kodak, Rochester, NY) for 5 min, washed in  $20^{\circ}C$  tap water for 20 min, dipped in Kodak photoflo 200 (Kodak, Rochester, NY) and air dried.

## RESULTS

Sporozoite Penetration of Cultured MDBK Cells

Ultrastructural studies on the process of in vitro host cell penetration by E. bovis sporozoites, with specimens postfixed in OsO<sub>4</sub> solutions containing ruthenium red after a 5 or 10 min reaction interval, revealed that the plasmalemma of the host cell was initially invaginated by the sporozoite (Figs. 1, 2). Since the electron dense ruthenium red stain was limited only to the exposed plasmalemmae of host cells and sporozoites alike, the staining of host cell membranes that are adjacent to penetrating sporozoites indicated that the penetration process in these situations was not complete by the time of fixation (Figs. 1, 2). In support of these findings, no ruthenium red was seen on the plasmalemmae of sporozoites which were entirely intracellular after 5 or 10 min (Figs. 2-5). Likewise, staining of host cell membrane segments that were closely associated with parasites that were entirely intracellular did not occur (Fig. 2). Intracellular staining by ruthenium red was never observed in these experiments.

Further electron microscopical observations of these preparations indicated that the host cell plasmalemma did not remain around intracellular sporozoites for an

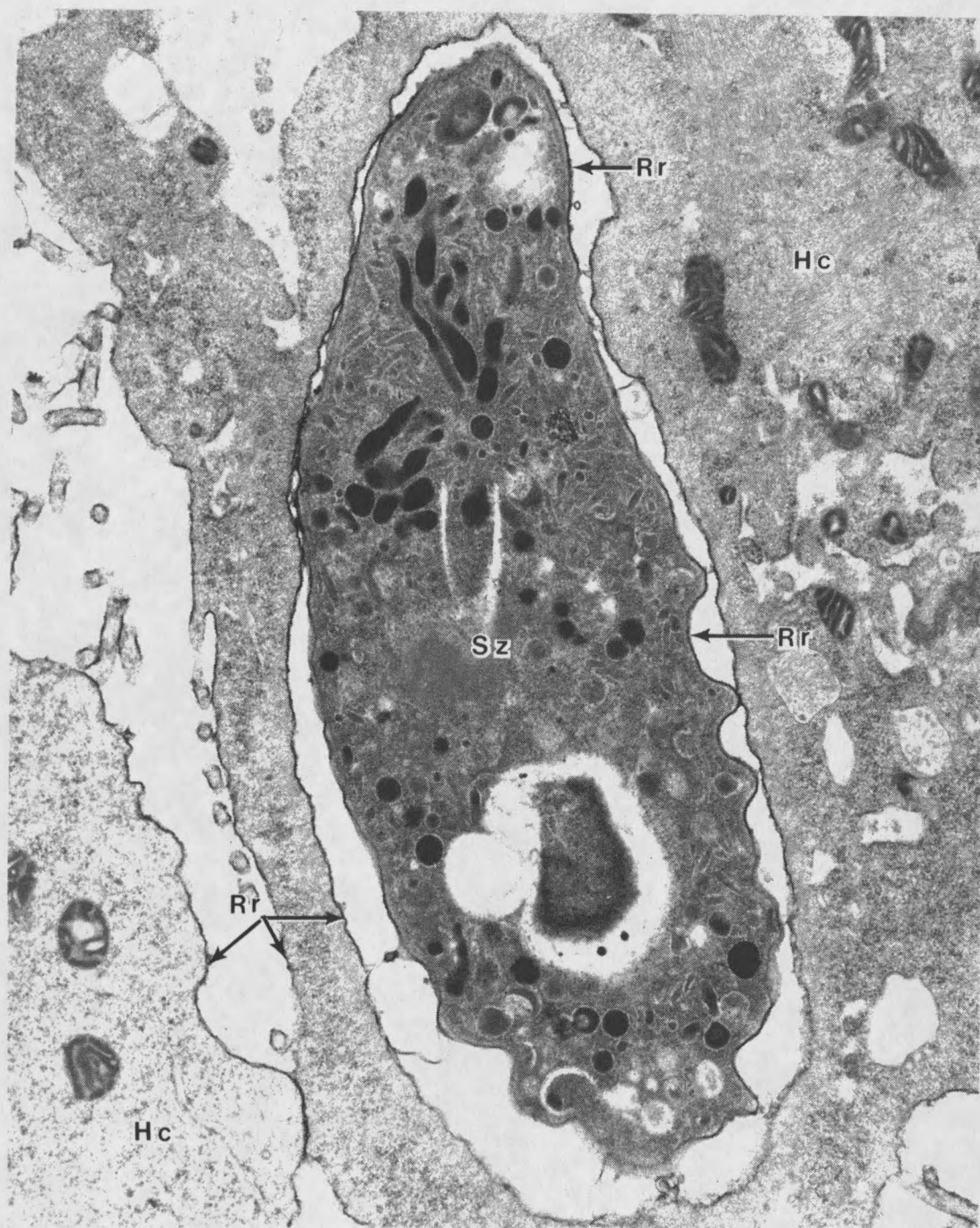


Fig. 1. *Eimeria bovis* sporozoite (Sz) in the process of penetrating a MDBK cell. The ruthenium red (Rr) stain is limited to the sporozoite plasmalemma and the intact host cell surfaces. Hc, host cell. Postfixed at 10 min. X 19,800.

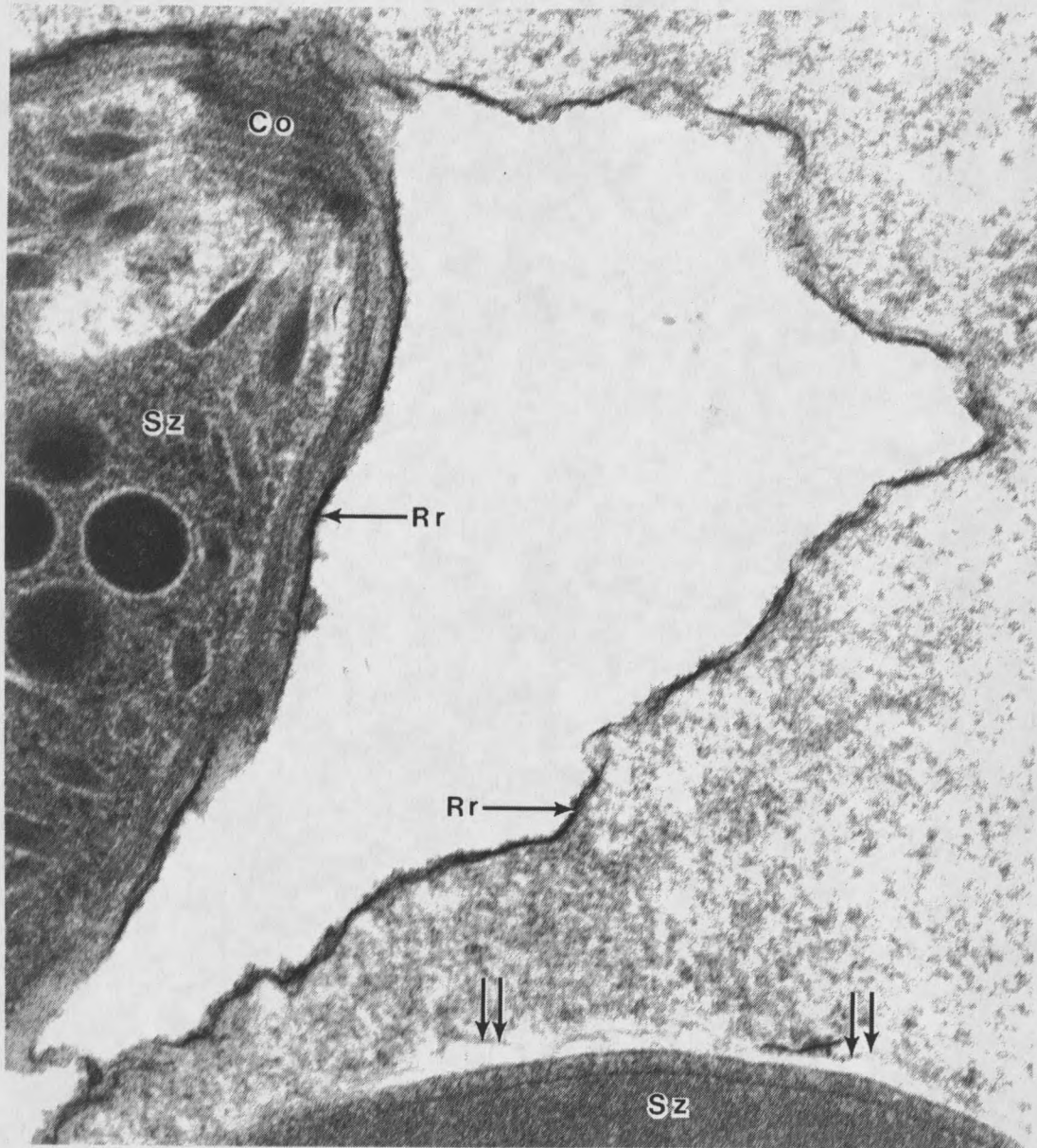


Fig. 2. High magnification transmission electron micrograph showing portions of two sporozoites (Sz) of *E. bovis*. Note ruthenium red (Rr) on plasmalemmae of apical region of penetrating sporozoite (upper left) and of MDBK cell. Sporozoite at bottom of figure is surrounded by unstained segments of host cell plasmalemma (double arrow) indicating that this sporozoite was completely intracellular at time of fixation. Co, conoid. Postfixed at 10 min. X 78,000.

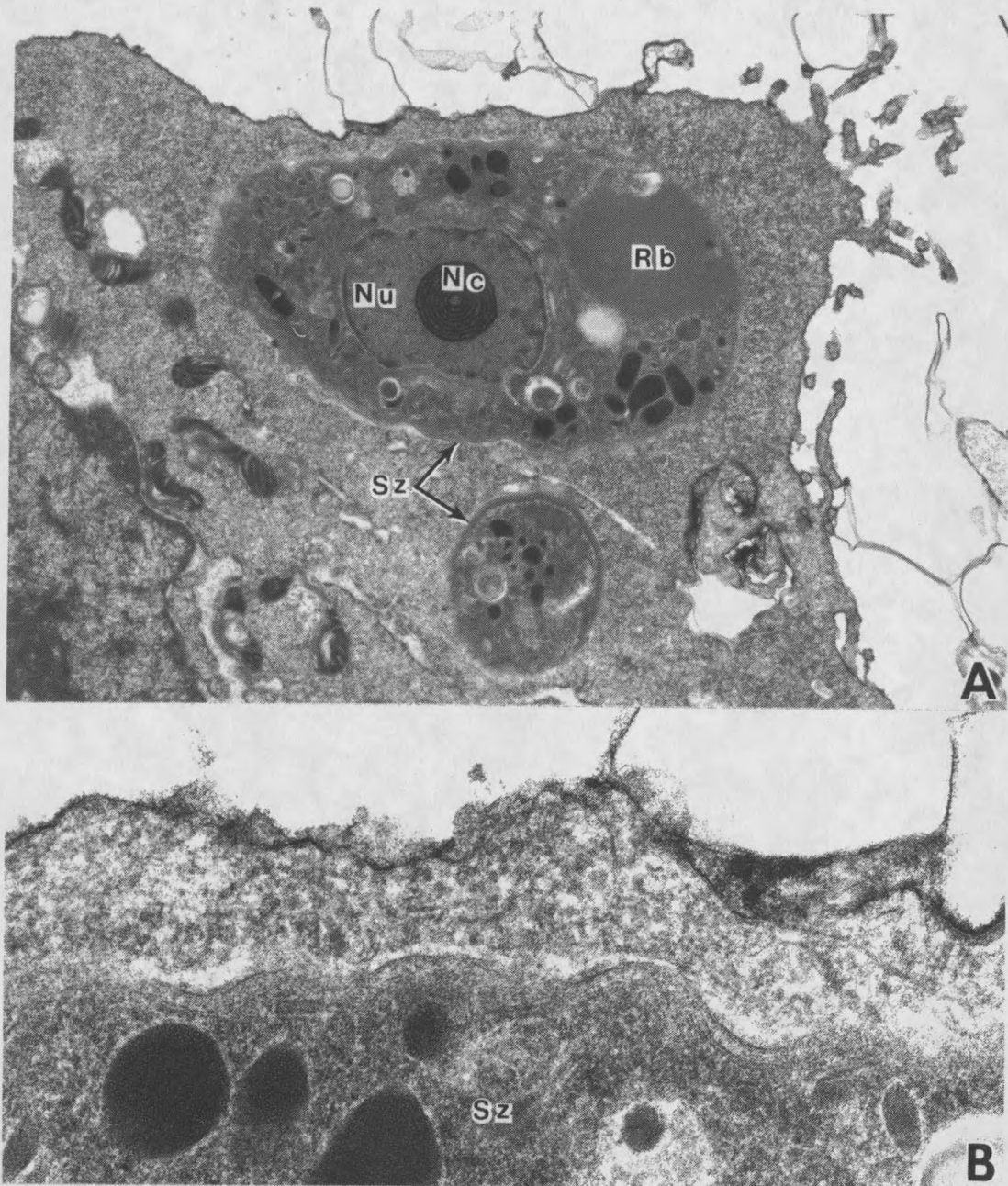


Fig. 3. A. Low magnification of two sporozoites (Sz) of *E. bovis* within a MDBK cell. Note the electron-dense ruthenium red on the exterior surface of the plasmalemma of the host cell. Nu, sporozoite nucleus; Nc, sporozoite nucleolus; Rb, refractile body. Postfixed at 10 min. X 15,000. B. Higher magnification of portion of A showing interface between sporozoite (Sz) and host cell. X 78,000.

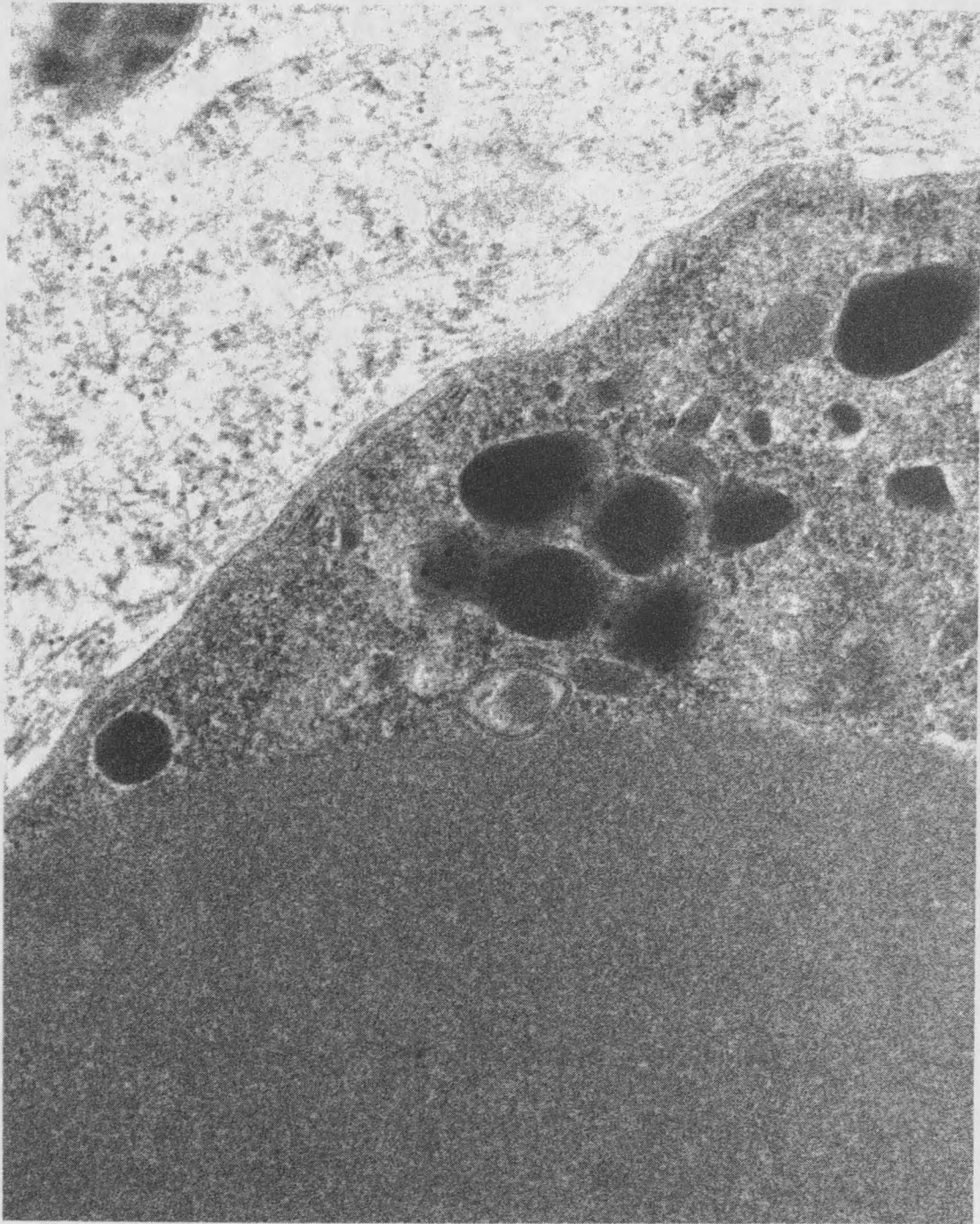


Fig. 4. High magnification of sporozoite of E. bovis free in the cytoplasm of a MDBK cell. Postfixed at 10 min. X 78,000.

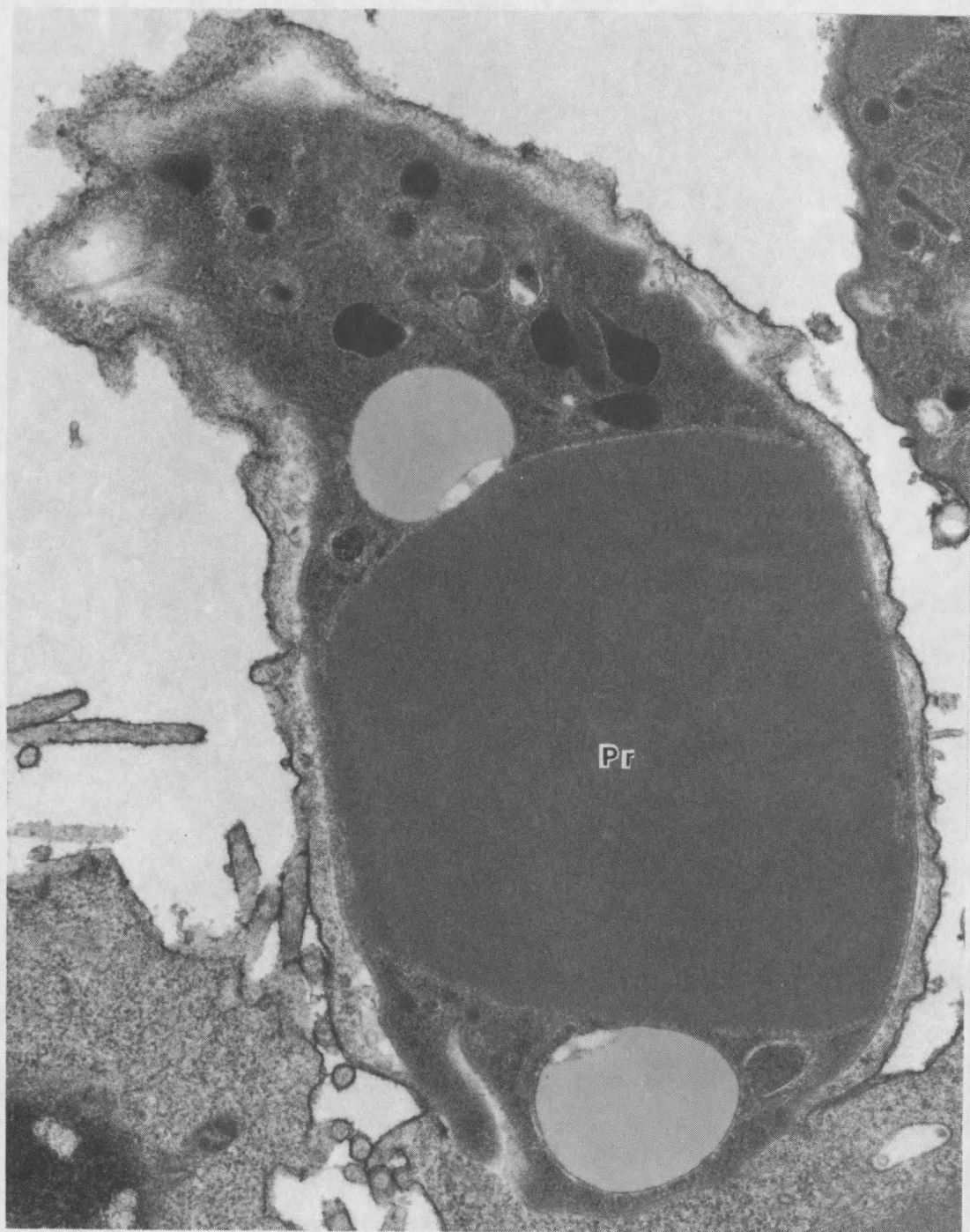


Fig. 5. Sporozoite in the process of exiting from a MDBK cell. Note that the sporozoite is carrying an envelope of host cell cytoplasm and ruthenium red-stained plasmalemma with it. Pr, posterior refractile body. Postfixed at 10 min. X 30,000.

appreciable amount of time, since up to 56% of these parasites were seen to reside free in the cytoplasm of host cells after a 5 or 10 min reaction time before fixation (Figs. 3, 4). Occasionally, intracellular sporozoites were seen in the process of exiting from host cells (Fig. 5). These parasites were not surrounded by a membrane-bound vacuole and appeared to carry host cell cytoplasm and plasmalemma with them as they left the host cell (Fig. 5). No ultrastructural changes in the sporozoite conoid, plasmalemma or rhoptries were observed during parasite penetration of host cells.

#### Parasite-specific IgG Titrations

IFA assays with serum from two noninfected calves showed little or no fluorescence with acetone-fixed E. bovis sporozoites. Parasite-specific IgG was first detected in one of these two calves 1 week after oocyst inoculation and the other became positive at 2 weeks (Figs. 6, 7). Peak titers of 40 were obtained from the sera of both calves during weeks 3 and 4 (Fig. 6). At 5 weeks, the IgG titers in both animals had decreased to pre-inoculation levels of 10 (Fig. 6).

Six weeks after the initial inoculation, both calves were given a per os inoculation of  $5 \times 10^4$  oocysts. Their parasite-specific IgG titers increased rapidly within two weeks after challenge reaching a maximum titer of 160 which

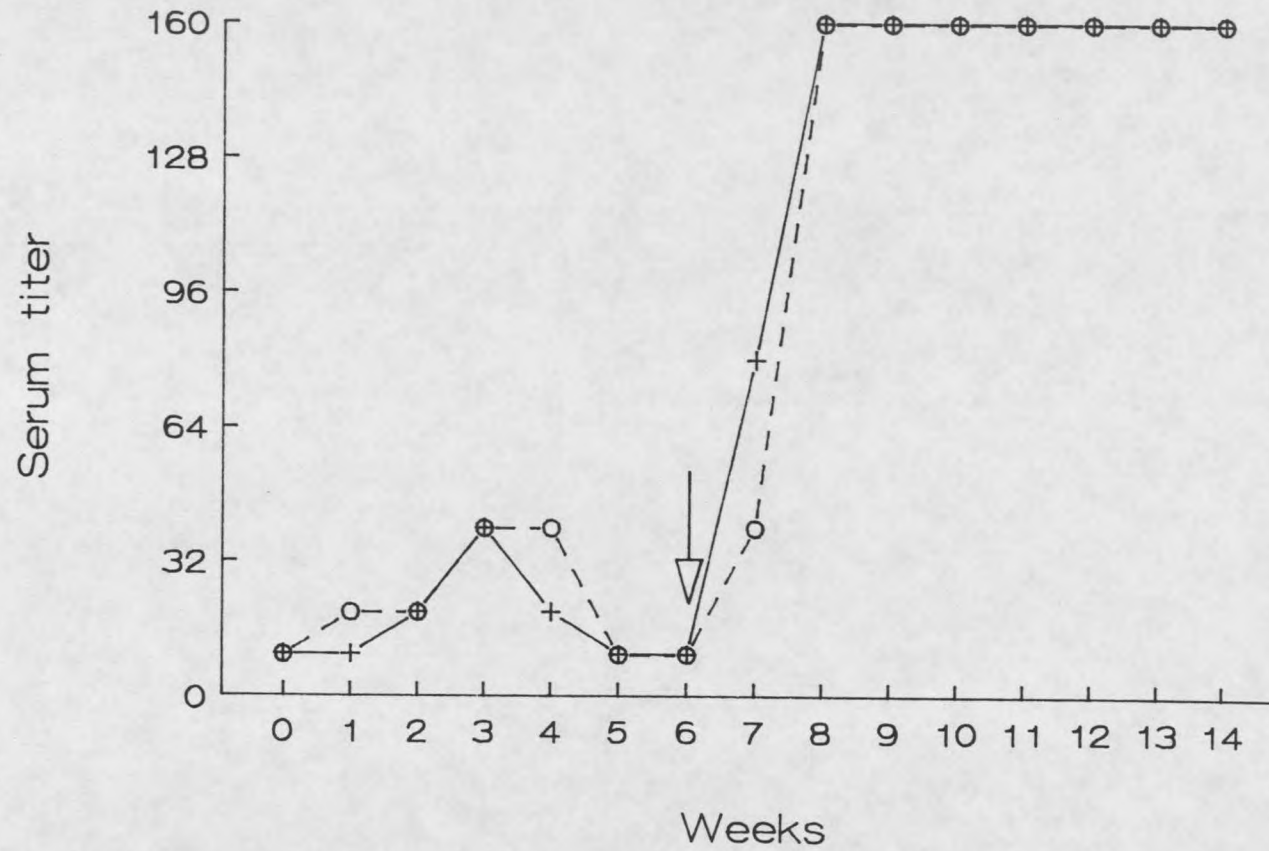


Fig. 6. Immunofluorescent assay titrations of parasite-specific IgG with sera obtained from two calves (+, o). Each calf was initially inoculated (0) with  $3.5 \times 10^4$  oocysts followed 6 weeks later by a challenge inoculation of  $5 \times 10^4$  oocysts (arrow).

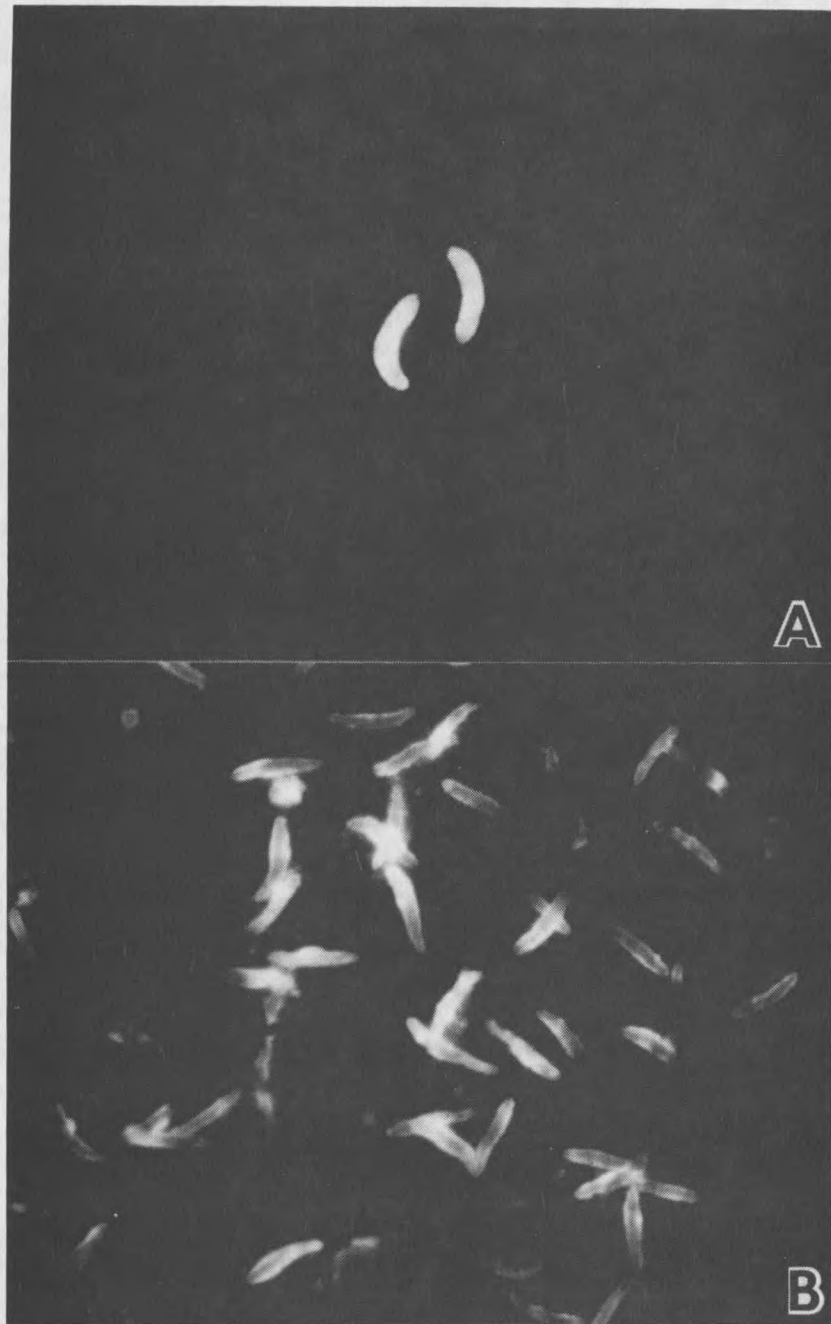


Fig. 7. Sporozoites (A) and merozoites (B) exhibiting whole cell immunofluorescence. Treatment: Pre-fixed, immune serum, fluorescein-conjugated rabbit antiovine IgG. A, X 900; B, X 1,400.

then persisted for an additional 6 weeks (Fig. 6). IgG in the sera obtained from these calves 3 weeks after the initial per os inoculation also reacted with first-generation merozoites by IFA assays (Fig. 7).

#### Patency Period

Both calves used in the parasite-specific IgG titration experiment shed oocysts in their feces at 17 to 30 days after the initial inoculation of  $3.5 \times 10^4$  oocysts. However, peak oocyst discharge occurred at 18 to 23 days after inoculation and was accompanied by hemorrhagic diarrhea. At 19 days after inoculation,  $185 \times 10^6$  oocysts were collected from both calves whereas only  $22 \times 10^6$  oocysts were collected at 23 days. On 18 to 20 days after the initial per os inoculation, numerous E. bovis oocysts could be seen per gram of wet feces that was recovered from each calf during the primary infection. Conversely, after obtaining fecal samples from each calf on days 15 through 24 following a per os challenge of  $5 \times 10^4$  oocysts (Fig. 6), only a single E. bovis oocyst was found per gram of wet feces from each calf on day 18. This day corresponds closely to the period of peak oocyst discharge during the primary infection. After the challenge inoculation, both calves exhibited no clinical illness.

### Monoclonal Antibodies

The first 7 MAbs listed in Table 1 were provided by the Immunology Laboratory of the Department of Veterinary Science, whereas 15 other MAbs were produced in the EM Laboratory, 5 of which are also included in Table 1. All of the MAbs were elicited against whole E. bovis sporozoites and were further characterized in the EM Laboratory. All 12 of the MAbs used in this study were found to be subclasses of murine IgG immunoglobulin by ELISA and demonstrated various patterns of fluorescence on acetone-fixed sporozoites by IFA assays (Table 1; Fig. 8). The ability of EbS14 to cross-react with merozoites was not precisely determined due to ambiguous IFA results. EbS7, 15 and 16 reacted with acetone-fixed but not with live sporozoites and first-generation merozoites (Table 1), indicating that both stages contain common internal antigens. None of the other MAbs tested cross-reacted against live or acetone-fixed merozoites.

Except for the EbS16, none of the MAbs tested appeared to cross-react with other bovine eimerian sporozoites which were sometimes present in sporozoite preparations of E. bovis (Fig. 8). EbS16 cross-reacted with acetone-fixed sporozoites which were smaller and had fewer and smaller refractile bodies than those of E. bovis. These smaller

sporozoites appeared similar to those of E. ellipsoidalis and E. zuernii (Dr. C.A. Speer, personal communication).

Table 1. Immunoglobulin subclass and indirect fluorescent antibody assay of various monoclonal antibodies with sporozoites and merozoites of E. bovis.

| Anti-sporozoite<br>MAb <sup>a</sup> | IgG<br>subclass   | IFA sporozoite <sup>b</sup> |                   | IFA merozoite <sup>c</sup> |                   |
|-------------------------------------|-------------------|-----------------------------|-------------------|----------------------------|-------------------|
|                                     |                   | Fixed <sup>d</sup>          | Live <sup>e</sup> | Fixed <sup>d</sup>         | Live <sup>e</sup> |
| EbS1                                | IgG <sub>1</sub>  | Whole                       | -                 | -                          | -                 |
| EbS7                                | IgG <sub>1</sub>  | Whole                       | -                 | +                          | -                 |
| EbS9                                | IgG <sub>1</sub>  | Apical                      | Whole             | -                          | -                 |
| EbS11                               | IgG <sub>2a</sub> | Apical                      | Whole             | -                          | -                 |
| EbS14                               | IgG <sub>2a</sub> | Whole                       | -                 | ?                          | -                 |
| EbS15                               | IgG <sub>2a</sub> | Whole                       | -                 | +                          | -                 |
| EbS16                               | IgG <sub>3</sub>  | Whole                       | -                 | +                          | -                 |
| EbS17                               | IgG <sub>2a</sub> | Speckled                    | -                 | -                          | -                 |
| EbS18                               | IgG <sub>2a</sub> | RF body                     | -                 | -                          | -                 |
| EbS20                               | IgG <sub>2a</sub> | Whole                       | -                 | -                          | -                 |
| EbS22                               | IgG <sub>2a</sub> | Speckled                    | -                 | -                          | -                 |
| EbS25                               | IgG <sub>2a</sub> | Whole                       | -                 | -                          | -                 |

<sup>a</sup>Monoclonal antibody.

<sup>b</sup>IFA pattern exhibited by sporozoites. Whole indicates immunofluorescence of entire sporozoite whereas apical, speckled and RF body (refractile body) denote regional immunofluorescence of sporozoites.

<sup>c</sup>Cross-reactivity of anti-sporozoite MAbs with invitro-produced first-generation merozoites.

<sup>d</sup>Sporozoites and merozoites were prefixed in acetone before exposure to MAb-containing ascites or culture medium.

<sup>e</sup>Sporozoites were postfixed in 0.2% glutaraldehyde after exposure to heat-inactivated MAb-containing ascites or culture medium.

EbS9 and EbS11 reacted with the apex of acetone-fixed sporozoites and with whole sporozoites in the live IFA (Table 1; compare Fig. 9 with Fig. 10), but did not react with live nor acetone-fixed first-generation merozoites. In the live IFA, EbS9 and EbS11 caused a low degree of sporozoite agglutination (Fig. 10). Also in live IFA, EbS9

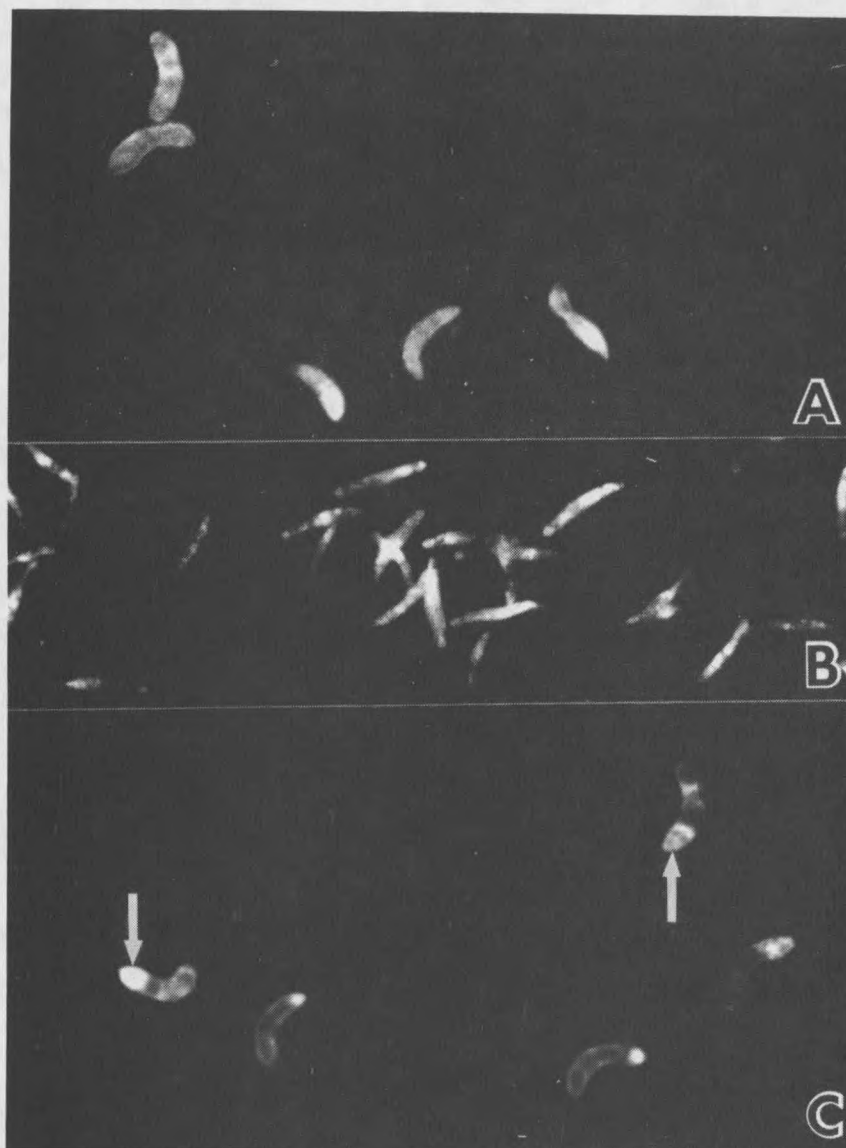


Fig. 8. Photomicrographs showing immunofluorescence patterns of MAbs on sporozoites and merozoites. All specimens were prefixed and treated with ascites containing MAb followed by fluorescein-conjugated goat antimouse IgG. A. Sporozoites exhibiting whole cell fluorescence after exposure to EbS15. X 900. B. Cross-reaction of EbS15 with merozoites demonstrates a speckled fluorescent pattern. X 900. C. Sporozoites exhibiting intense apical fluorescence (arrows) following exposure to EbS11. X 900.

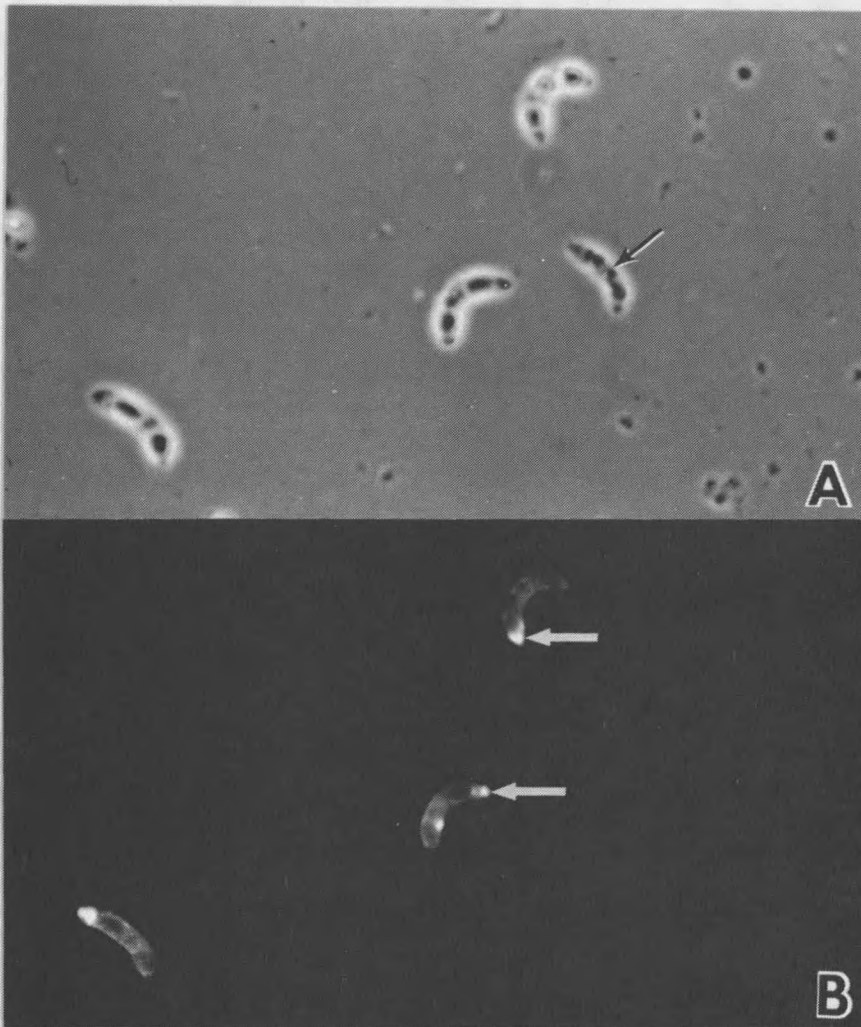


Fig. 9. A. Phase-contrast photomicrograph of several sporozoites of *E. bovis* and one sporozoite of *E. ellipsoidalis* or *E. zuernii* (arrow). X 900. B. Photomicrograph of IFA of the same specimens in A showing fluorescence with EbS9 in sporozoites of *E. bovis* but not in that of *E. ellipsoidalis* or *E. zuernii*. Note intense apical region fluorescence of sporozoites of *E. bovis* (arrows). Treatment: Prefixed, MAb-containing ascites, fluorescein-conjugated goat antimouse IgG. X 900.



Fig. 10. Photomicrographs showing immunofluorescence by live IFA with EbS9 on a sporocyst and sporozoites of *E. bovis*. A. Whole cell fluorescence of agglutinated sporozoites. X 1,100. B. Fluorescence of sporocyst and sporozoite within sporocyst; note that sporocyst wall fluoresces intensely at one pole (arrow) near gap created by dissolution of Stieda body. Sw, sporocyst wall. X 1,100. Treatment: A and B; MAb-containing ascites, postfixed, fluorescein-conjugated goat antimouse IgG.

and EbS11 reacted with the sporocyst wall especially at one pole of the sporocyst near the gap created by dissolution of the Stieda body (Fig. 10). EbS9 and EbS11 reacted with sporozoites within sporocysts with no Stieda body but did not react with the sporocyst wall or sporozoites in intact sporocysts (i.e. the Stieda body had not undergone dissolution in the excysting fluid).

#### Sporozoite Penetration Inhibition Assays

At 24 hr after sporozoite inoculation (ASI), cultures of MDBK cells inoculated with EbS9 or EbS11 pretreated sporozoites contained significantly fewer ( $P \leq 0.05$ ) intracellular sporozoites (79 or 73% decrease, respectively) than did cultures that were inoculated with Ag8 pretreated sporozoites (Figs. 11, 12; Table 2). Also, at 24 hr ASI, there were no significant differences in mean numbers of intracellular sporozoites in MDBK cell cultures inoculated with sporozoites that had been pretreated with EbS7, EbS14, EbS15 or Ag8 (Table 2; compare Figs. 12 and 13). No significant differences in mean numbers of intracellular sporozoites were detected in cultures in which the MDBK cells (and not the sporozoites) had been pretreated with EbS9, EbS11 or DMEM (Table 3).

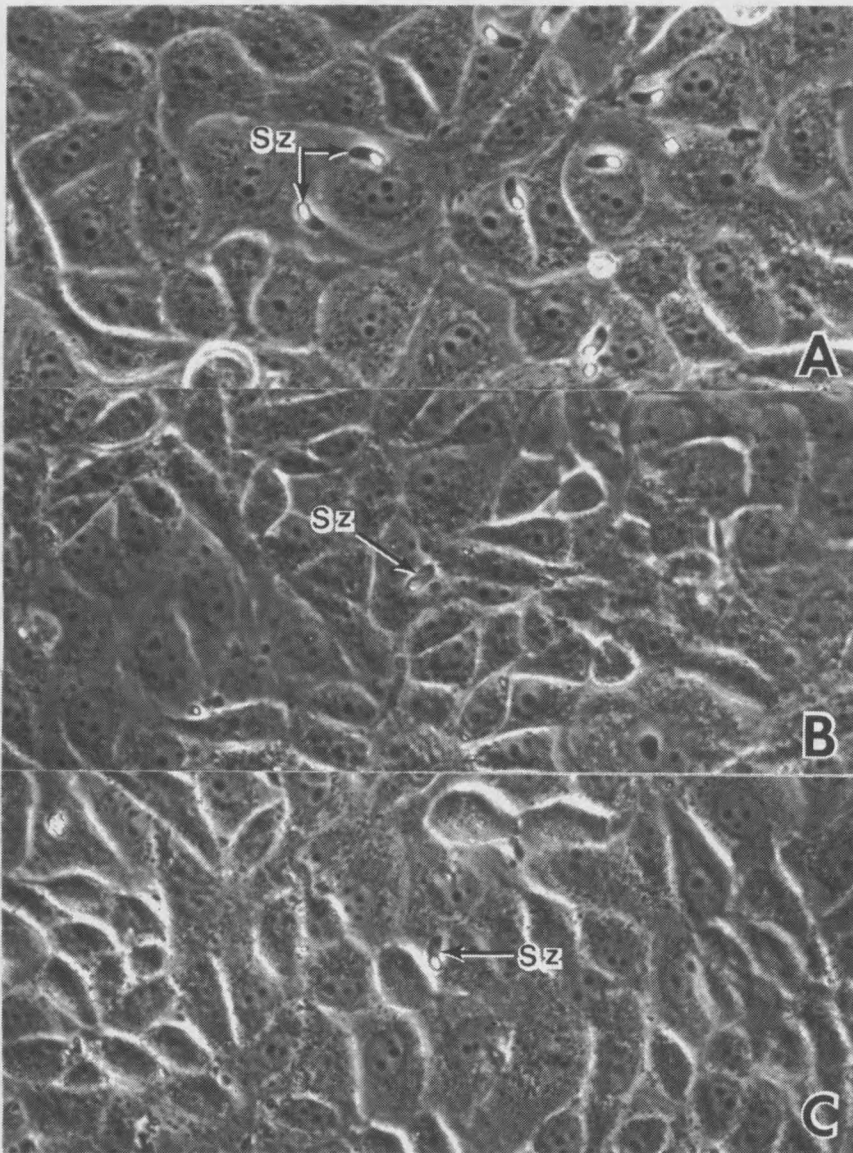


Fig. 11. Phase-contrast photomicrographs of live *E. bovis* sporozoites (Sz) in MDBK cells 24 hr ASI. A. Several intracellular sporozoites that had been treated previously with Ag8 for 30 min at RT before inoculation into MDBK cell cultures. X 400. B. Relatively few intracellular sporozoites are present in these MDBK cells; sporozoites were pretreated with EbS9 for 30 min at RT. X 400. C. Similar to B, except that the sporozoites were pretreated with EbS11. X 400.

Table 2. Effects of monoclonal antibodies on penetration of MDBK cells by sporozoites of *E. bovis*.<sup>a</sup>

| Treatment <sup>b</sup> | IFA titer | Number of intracellular sporozoites (24 hr ASI) <sup>c</sup> |
|------------------------|-----------|--------------------------------------------------------------|
| EbS9 <sup>d</sup>      | 20        | 39+5 <sup>f</sup>                                            |
| EbS11 <sup>d</sup>     | 20        | 50+5 <sup>f</sup>                                            |
| Ag8 <sup>e</sup>       | -         | 185+12                                                       |
| EbS7 <sup>d</sup>      | 20        | 46+9 <sup>g</sup>                                            |
| EbS14 <sup>d</sup>     | 10        | 54+9 <sup>g</sup>                                            |
| EbS15 <sup>d</sup>     | 10        | 44+11 <sup>g</sup>                                           |
| Ag8 <sup>e</sup>       | -         | 46+10 <sup>g</sup>                                           |

<sup>a</sup>Results of two experiments.<sup>b</sup>Before inoculation into cell cultures, sporozoites were treated for 30 min at RT with culture medium with or without monoclonal antibodies.<sup>c</sup>Sample size, 4 counts. Values are  $\bar{x} \pm$  standard deviation.<sup>d</sup>Monoclonal antibodies.<sup>e</sup>Culture medium from unfused P3-X63-Ag8.653 (Ag8) myeloma cell culture.<sup>f</sup>Significantly different ( $P \leq 0.05$ ) from Ag8 control.<sup>g</sup>Not significantly different ( $P > 0.05$ ).Table 3. Effects of monoclonal antibody treatment of MDBK cells on penetration by sporozoites of *E. bovis*.

| Treatment <sup>a</sup> | IFA titer | Number of intracellular sporozoites (24 hr ASI) <sup>b</sup> |
|------------------------|-----------|--------------------------------------------------------------|
| EbS9 <sup>c</sup>      | 20        | 286+27 <sup>e</sup>                                          |
| EbS11 <sup>c</sup>     | 20        | 278+17 <sup>e</sup>                                          |
| DMEM <sup>d</sup>      | -         | 280+30 <sup>e</sup>                                          |

<sup>a</sup>MDBK cell cultures were previously treated with culture medium with or without monoclonal antibodies for 30 min at RT before inoculation of untreated sporozoites.<sup>b</sup>Sample size, 4 counts. Values are  $\bar{x} \pm$  standard deviation.<sup>c</sup>Monoclonal antibodies.<sup>d</sup>Dulbecco's Modified Eagle Medium.<sup>e</sup>Not significantly different ( $P > 0.05$ ).

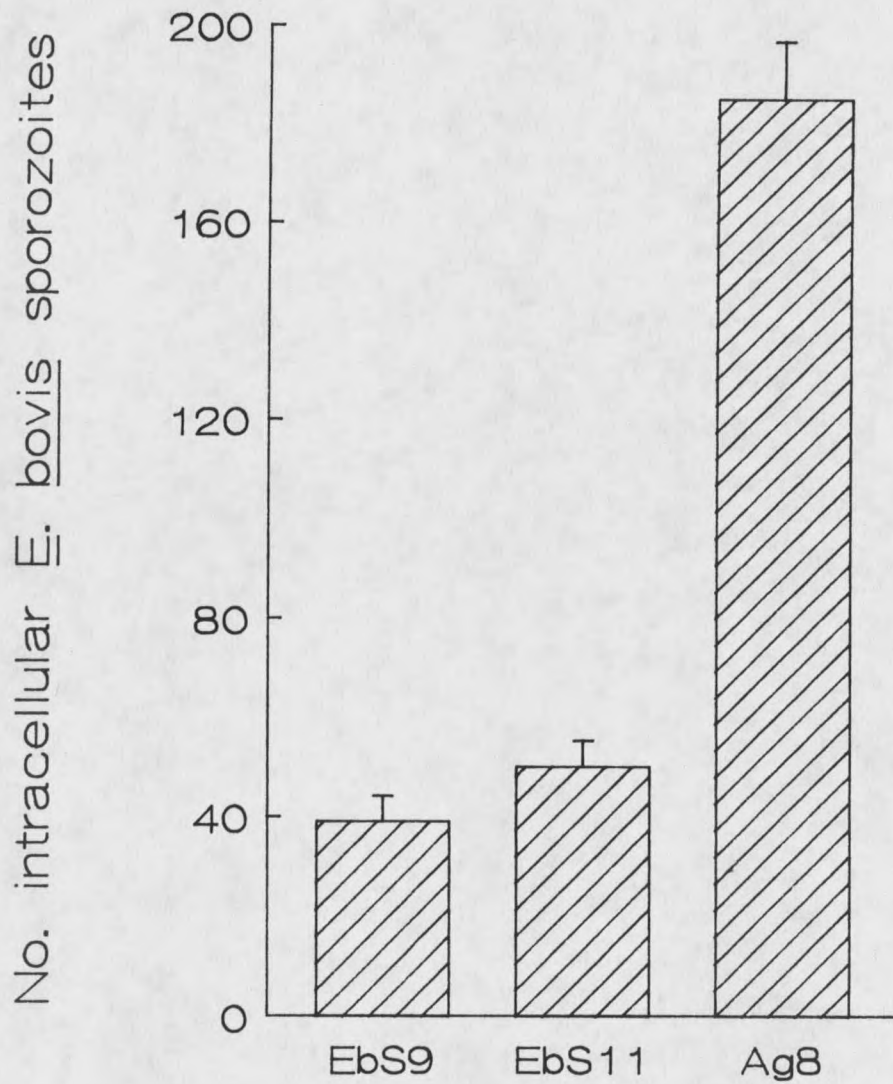


Fig. 12. Effects of pretreating sporozoites of E. bovis with surface-reactive MAbs 24 hr ASI of MDBK cells.

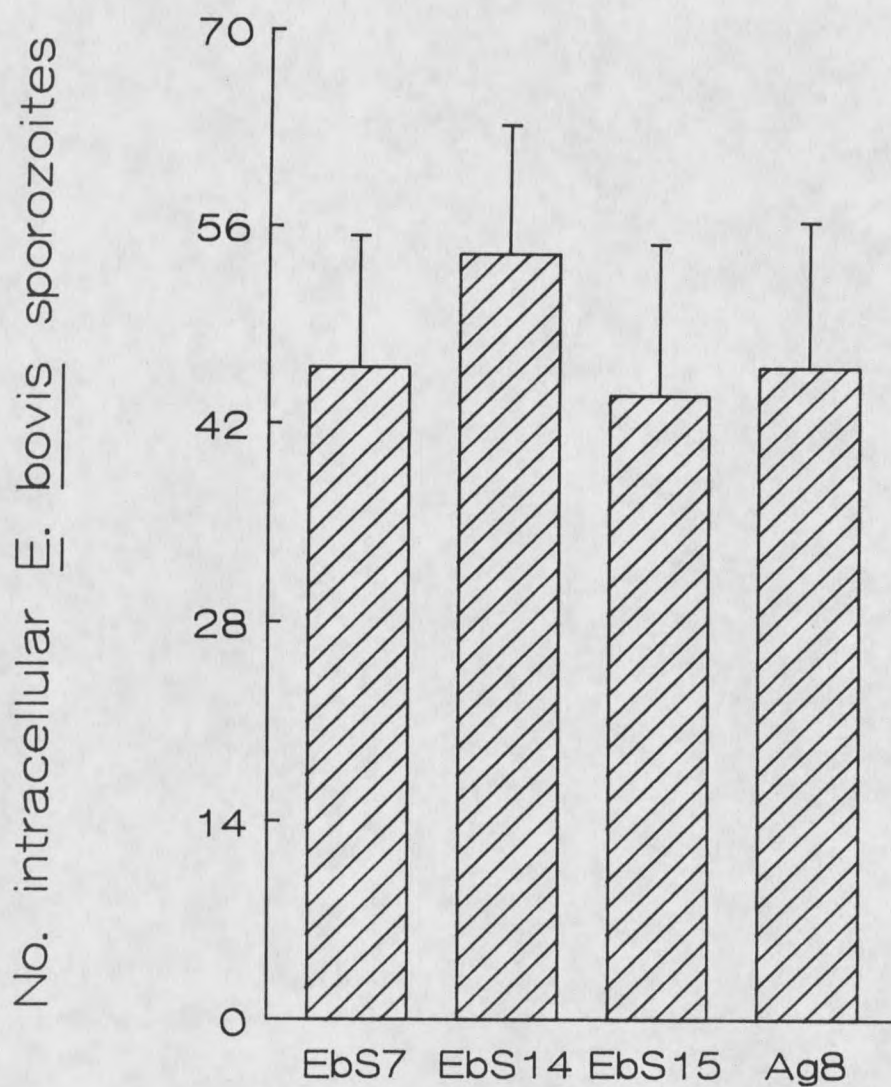


Fig. 13. Effects of pretreating sporozoites of *E. bovis* with nonsurface-reactive MAbs 24 hr ASI of MDBK cells.

Table 4 shows at 24 hr ASI the relative mean numbers of intracellular E. bovis sporozoites that were pretreated with various dilutions of heat-inactivated AS, IS, NS or DMEM before inoculation into MDBK cell cultures.

Table 4. Effects of pretreating sporozoites of E. bovis with various sera on their ability to penetrate MDBK cells.

| Treatment <sup>a</sup>     | Dilution<br>(PBS, 0.15M) | Number of<br>intracellular<br>sporozoites<br>(24 hr ASI) <sup>b</sup> |
|----------------------------|--------------------------|-----------------------------------------------------------------------|
| AS<br>(IFA titer<br>= 160) | 1/8<br>1/2<br>0          | 79+12 <sup>c, d</sup><br>43+7 <sup>e</sup><br>65+7 <sup>d</sup>       |
| IS<br>(IFA titer<br>= 80)  | 1/4<br>1/2<br>0          | 108+27 <sup>c</sup><br>106+9 <sup>c</sup><br>106+16 <sup>c</sup>      |
| NS<br>(IFA -)              | 0                        | 86+9 <sup>c, d</sup>                                                  |
| DMEM<br>(IFA -)            | 0                        | 108+11 <sup>c</sup>                                                   |

<sup>a</sup>Sporozoites were pretreated with different dilutions of antiserum (AS), immune serum (IS), normal serum (NS) or serum free culture medium (DMEM) for 30 min at RT before inoculation into cell cultures. All sera were heat-inactivated at 56°C for 30 min immediately before experimentation.

<sup>b</sup>Sample size, 4 counts. Values are  $\bar{x}$  + standard deviation.

<sup>c, d</sup>Not significantly different ( $P > 0.05$ ).

<sup>e</sup>Significantly different ( $P \leq 0.05$ ) from NS control.

There were no significant differences in the mean numbers of intracellular parasites in MDBK cell cultures that had been inoculated with sporozoites pretreated with diluted or undiluted IS, undiluted or a 1/8 dilution of AS,

NS or DMEM (Table 4; Fig. 14). However, in contrast to control cultures, significantly fewer intracellular sporozoites were present in MDBK cells in which the sporozoites had been pretreated with a 1/2 dilution of AS (Table 4; Fig. 14).

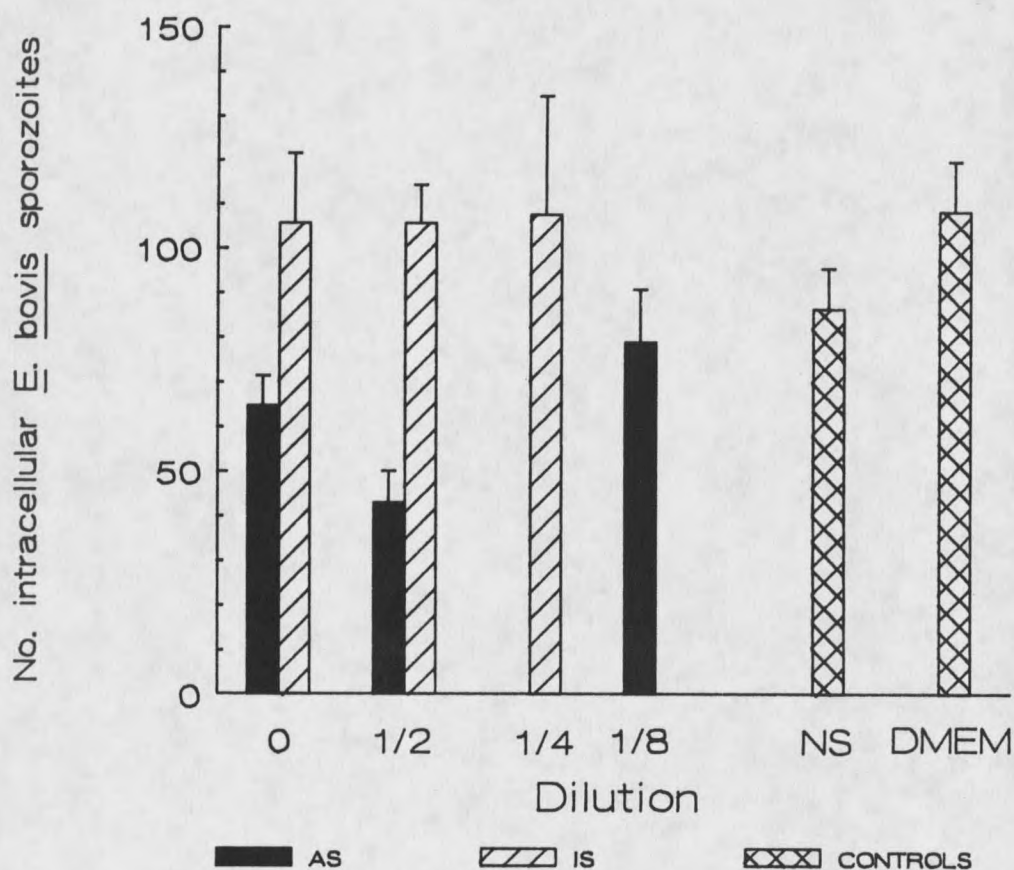


Fig. 14. Effects of various dilutions of antiserum (AS), immune serum (IS) or normal serum (NS) on the number of pretreated intracellular sporozoites of *E. bovis* 24 hr ASI of MDBK cells. The parasite-specific IgG titers of undiluted (0) AS and IS were 160 and 80, respectively, by IFA assay.

### Inhibition of Intracellular Development

Cultures of M617 cells inoculated with E. bovis sporozoites pretreated with EbS9 or EbS11, contained significantly fewer ( $P \leq 0.05$ ) first-generation meronts at 10 days ASI (89 or 94% decrease, respectively) than did M617 cultures inoculated with Ag8 or RPMI pretreated sporozoites (Table 5; Fig. 15). There were no significant differences in mean numbers of intracellular sporozoites among any of the cultures at 10 days ASI (Table 5). Significantly more meronts had developed at 10 days ASI in M617 cell cultures inoculated with sporozoites that had been pretreated with EbS11 with an IFA titer of 10 rather than of 20 (compare Tables 5 and 6; Figs. 15 and 16). Furthermore, pretreatment of sporozoites with EbS11 that had an IFA titer of 10 caused no significant differences in the mean numbers of intracellular meronts when compared to cultures that were inoculated with EbS7 pretreated sporozoites (Table 6; Fig. 16). Sporozoite pretreatment with EbS11 that had an IFA titer of 10 resulted in only a slight significant decrease in meront development 10 days ASI when compared to M617 cell cultures that were inoculated with EbS15 pretreated sporozoites (Table 6; Fig. 16).

Table 7 shows the relative mean numbers of intracellular first-generation meronts of E. bovis 10 days ASI of M617 cell cultures with sporozoites that were

Table 5. Effects of parasite-specific surface-reacting monoclonal antibodies on intracellular development of *E. bovis* sporozoites in M617 cells.

| Treatment <sup>a</sup> | IFA titer | Number of intracellular meronts (10 days ASI) <sup>b</sup> | Number of intracellular sporozoites (10 days ASI) <sup>b</sup> |
|------------------------|-----------|------------------------------------------------------------|----------------------------------------------------------------|
| EbS9 <sup>c</sup>      | 20        | 2+1 <sup>f</sup>                                           | 4+2 <sup>g</sup>                                               |
| EbS11 <sup>c</sup>     | 20        | 1+2 <sup>f</sup>                                           | 4+2 <sup>g</sup>                                               |
| Ag8 <sup>d</sup>       | -         | 18+3 <sup>g</sup>                                          | 4+2 <sup>g</sup>                                               |
| RPMI <sup>e</sup>      | -         | 18+6 <sup>g</sup>                                          | 6+2 <sup>g</sup>                                               |

<sup>a</sup>Sporozoites were pretreated with culture medium with or without monoclonal antibodies for 30 min at RT before inoculation into cell cultures.

<sup>b</sup>Sample size, 4 counts. Values are  $\bar{x} \pm$  standard deviation.

<sup>c</sup>Monoclonal antibodies.

<sup>d</sup>Culture medium from unfused P3-X63-Ag8.653 (Ag8) myeloma cell culture.

<sup>e</sup>RPMI 1640 culture medium

<sup>f</sup>Significantly different ( $P \leq 0.05$ ) from Ag8 control.

<sup>g</sup>Not significantly different ( $P > 0.05$ ) within parasite stage group.

Table 6. Effects of parasite-specific monoclonal antibodies on development of *E. bovis* sporozoites in M617 cells.

| Treatment <sup>a</sup> | IFA titer | Number of intracellular meronts (10 days ASI) <sup>b</sup> |
|------------------------|-----------|------------------------------------------------------------|
| EbS7 <sup>c</sup>      | 10        | 18+2 <sup>e, f</sup>                                       |
| EbS11 <sup>c</sup>     | 10        | 15+2                                                       |
| EbS15 <sup>c</sup>     | 20        | 20+4 <sup>d, f</sup>                                       |

<sup>a</sup>Sporozoites were pretreated with monoclonal antibodies for 30 min at RT and then inoculated into M617 cells.

<sup>b</sup>Sample size, 4 counts. Values are  $\bar{x} \pm$  standard deviation.

<sup>c</sup>Monoclonal antibodies.

<sup>d</sup>Significantly different ( $P \leq 0.05$ ) from EbS11.

<sup>e</sup>Not significantly different ( $P > 0.05$ ) from EbS11.

<sup>f</sup>Not significantly different ( $P > 0.05$ ).

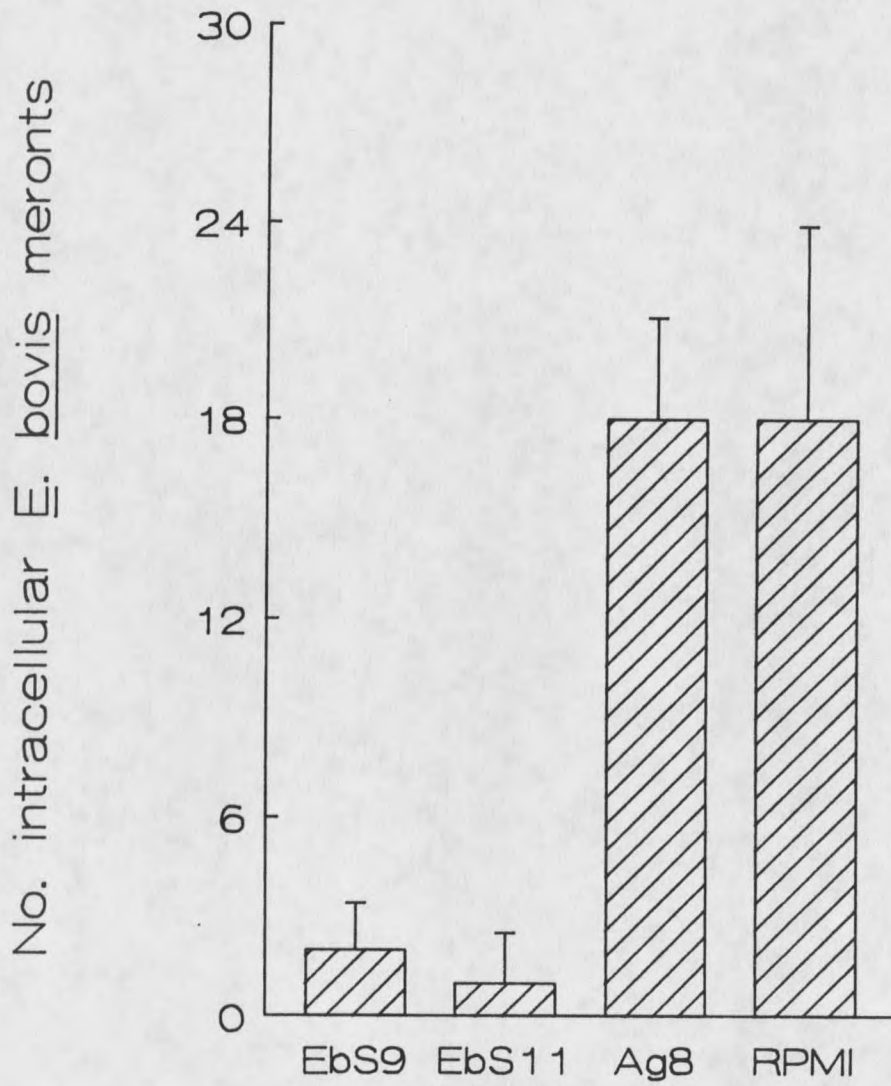


Fig. 15. Effects of surface-reactive MAbs on the number of intracellular *E. bovis* meronts 10 days ASI of M617 cells.

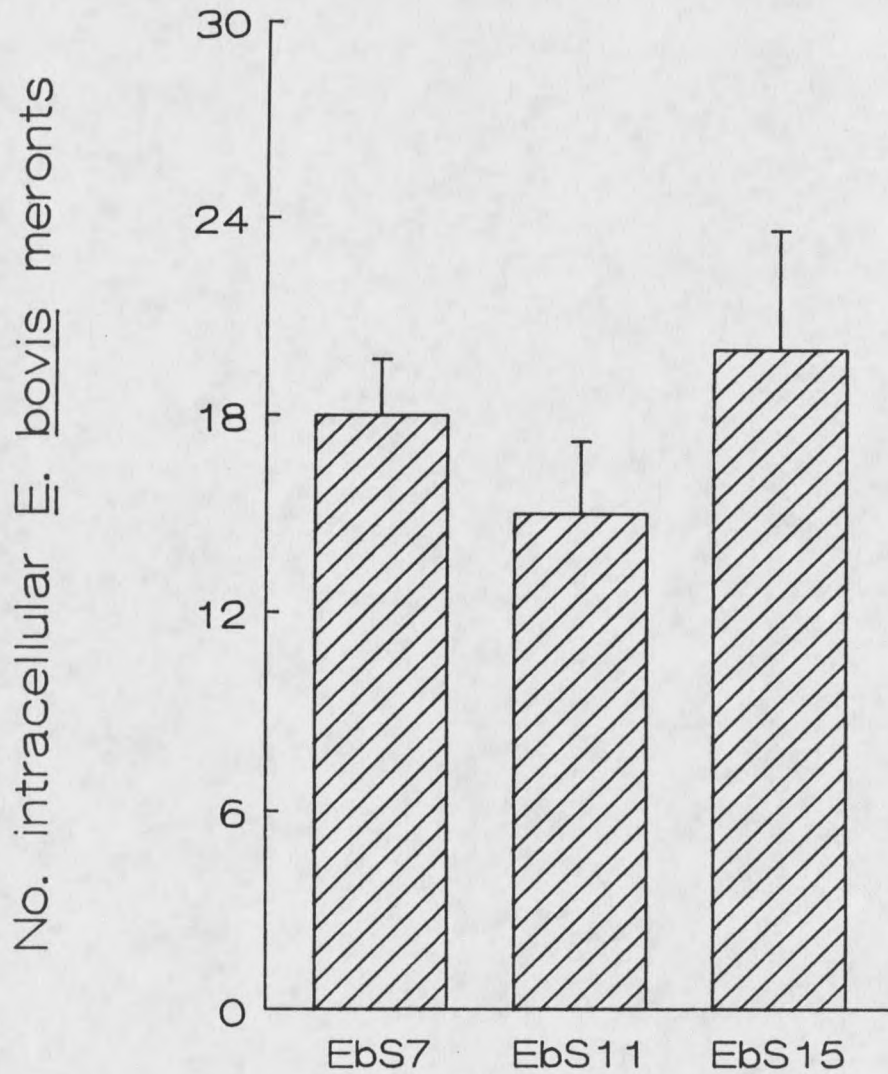


Fig. 16. Effects of parasite-specific MAbs on the number of intracellular meronts of *E. bovis* 10 days ASI of M617 cells.

pretreated with heat-inactivated AS, IS or NS. There were no significant differences in the mean numbers of meronts or intracellular sporozoites in cultures inoculated with AS or NS pretreated sporozoites (Table 7; Fig. 17).

Table 7. Effects of antiserum, immune serum and normal serum on development of E. bovis sporozoites in M617 cells.

| Treatment <sup>a</sup> | IFA titer | Number of intracellular meronts (10 days ASI) <sup>b</sup> | Number of intracellular sporozoites (10 days ASI) <sup>b</sup> |
|------------------------|-----------|------------------------------------------------------------|----------------------------------------------------------------|
| AS                     | 160       | 8+5 <sup>c</sup>                                           | 2+1 <sup>c</sup>                                               |
| IS                     | 40        | 0                                                          | 2+1 <sup>c</sup>                                               |
| NS                     | -         | 10+4 <sup>c</sup>                                          | 3+1 <sup>c</sup>                                               |

<sup>a</sup>Antiserum (AS), immune serum (IS) and normal serum (NS) were diluted 1/2 in serum free culture medium, filter-sterilized and reacted with sporozoites for 30 min at RT before sporozoite inoculation into M617 cell cultures. All sera were heat-inactivated at 56°C for 30 min immediately before experimentation.

<sup>b</sup>Sample size, 4 counts. Values are  $\bar{x} \pm$  standard deviation.

<sup>c</sup>Not significantly different ( $P > 0.05$ ) within parasite stage group.

#### Immunoelectron Microscopy

Nylon wool-purified E. bovis sporozoite preparations contained a few oocysts, oocyst walls and sporocysts in addition to sporozoites. The oocyst wall consisted of 2 layers, an electron-lucent inner layer and an electron-dense outer layer. Two closely applied membranes are interposed between the outer and inner layers. However, in most specimens only the inner layer of the oocyst wall was

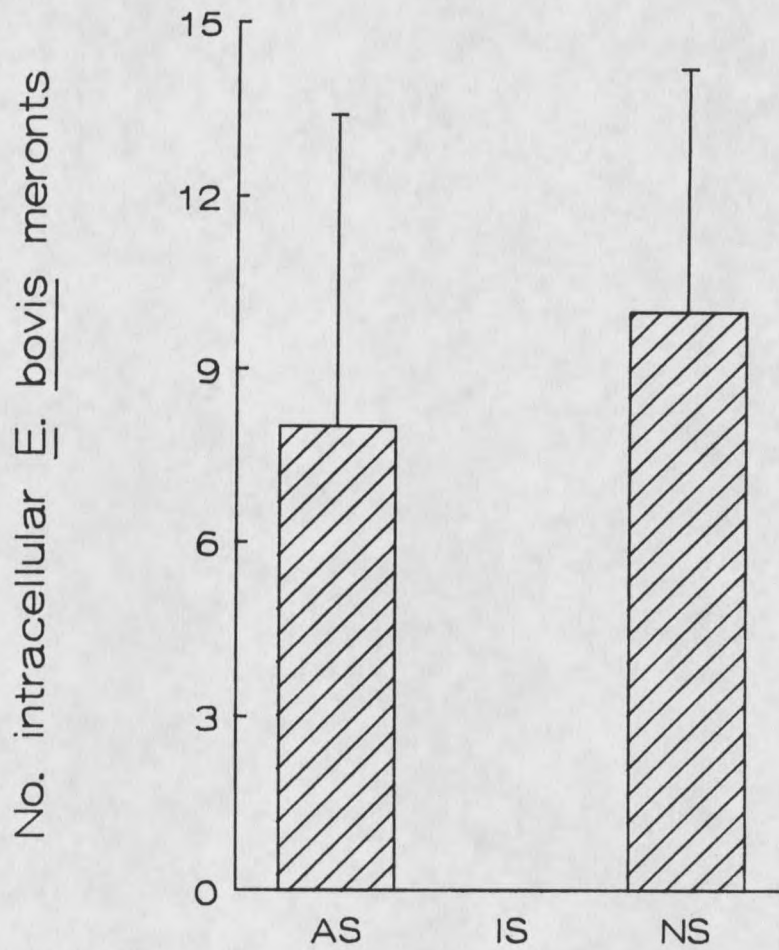


Fig. 17. Effects of antiserum (AS), immune serum (IS) or normal serum (NS) on the number of intracellular meronts of E. bovis 10 days ASI of M617 cells.

present because treatment with Clorox had removed the outer layer (Fig. 18). In oocyst walls in which the outer layer was missing, 1 or 2 of the interposed membranes were usually still attached to the outer surface of the inner layer (Fig. 18).

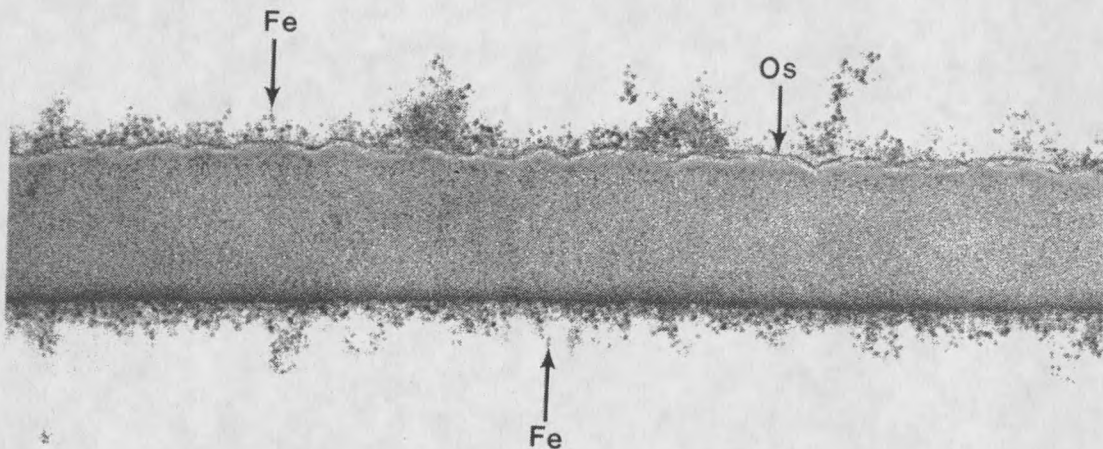


Fig. 18. Ultrastructural localization of parasite-specific bovine IgG by ferritin-conjugated antibovine IgG on both surfaces of the inner layer of an *E. bovis* oocyst wall. Note interposed membrane on the outer surface (Os) of the inner layer of the oocyst wall. Fe, ferritin. Treatment: Prefixed, immune serum, ferritin-conjugated rabbit antibovine IgG. X 78,000.

Sporozoites had all the organelles and inclusion bodies characteristically found in the Apicomplexa and *Eimeria* spp. such as a single nucleus, refractile bodies, conoid, polar and apical rings, micronemes, rhoptries, subpellicular

microtubules, mitochondria, Golgi complex, micropores, smooth and rough endoplasmic reticulum, ribosomes, amylopectin granules and lipid droplets (Figs. 19-27). Sporocysts were surrounded by a thin (~15 nm wide) electron-lucent sporocyst wall (Fig. 19).

Parasites prefixed in 0.15% glutaraldehyde, exposed to heat-inactivated AS or IS (IFA titers of 1280 and 160, respectively) and then ferritin-conjugated antiovine IgG antibody, exhibited similar ferritin binding patterns. Ferritin-conjugated antiovine IgG formed a relatively uniform layer on the inner and outer surface of the inner layer of the oocyst wall (Fig. 18).

Ferritin did not attach to intact sporocysts. However, in sporocysts in which the Stieda body had undergone dissolution in the presence of excysting fluid, ferritin formed a uniform layer on the inner surface of the sporocyst wall, outer surface of the sporocyst residuum and on the plasma-lemmae of sporozoites within sporocysts (Fig. 19). Slightly more ferritin attached to the inner surface of the sporocyst wall than to sporozoites within sporocysts (Fig. 19). Most free, prefixed sporozoites were covered by a uniform layer of ferritin, but in others ferritin was localized in patches (Figs. 20, 21). Occasionally, membrane-bound vesicles labelled with ferritin were found in close proximity to the surface of prefixed sporozoites (Fig. 20). Prefixed

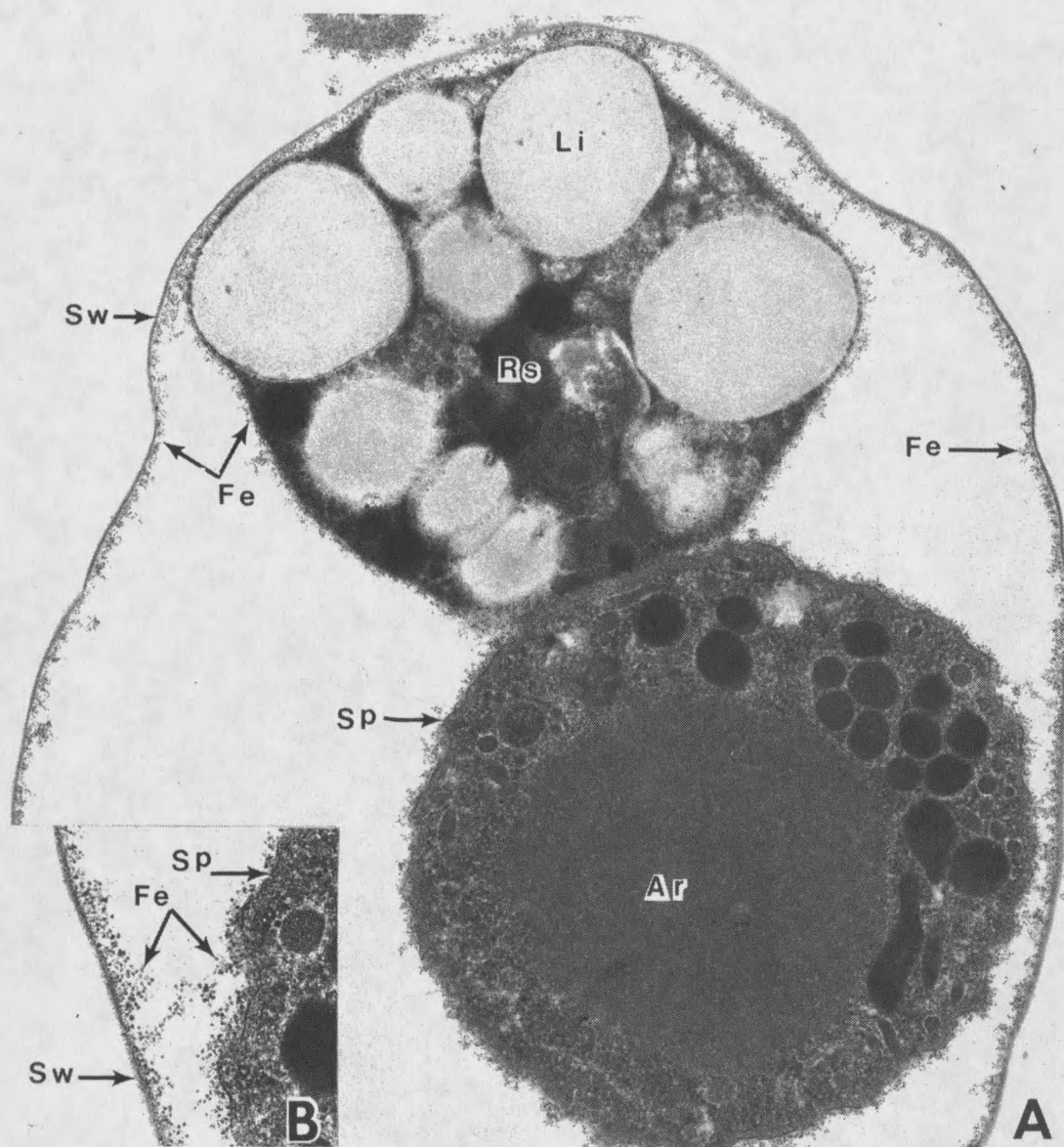


Fig. 19. Transmission electron micrographs of a sporocyst containing one sporozoite. A. Ferritin (Fe) has attached to the inner surface of the sporocyst wall (Sw), sporozoite plasmalemma (Sp) and sporocyst residuum (Rs). Ar, anterior refractile body of sporozoite; Li, lipid body. Treatment: Pre-fixed, immune serum, ferritin-conjugated rabbit anti-bovine IgG. X 30,000. B. Higher magnification of A showing abundant ferritin (Fe) on the sporozoite plasmalemma (Sp) and inner surface of the sporocyst wall (Sw). Note the absence of ferritin on the outer surface of the sporocyst wall. X 60,000.

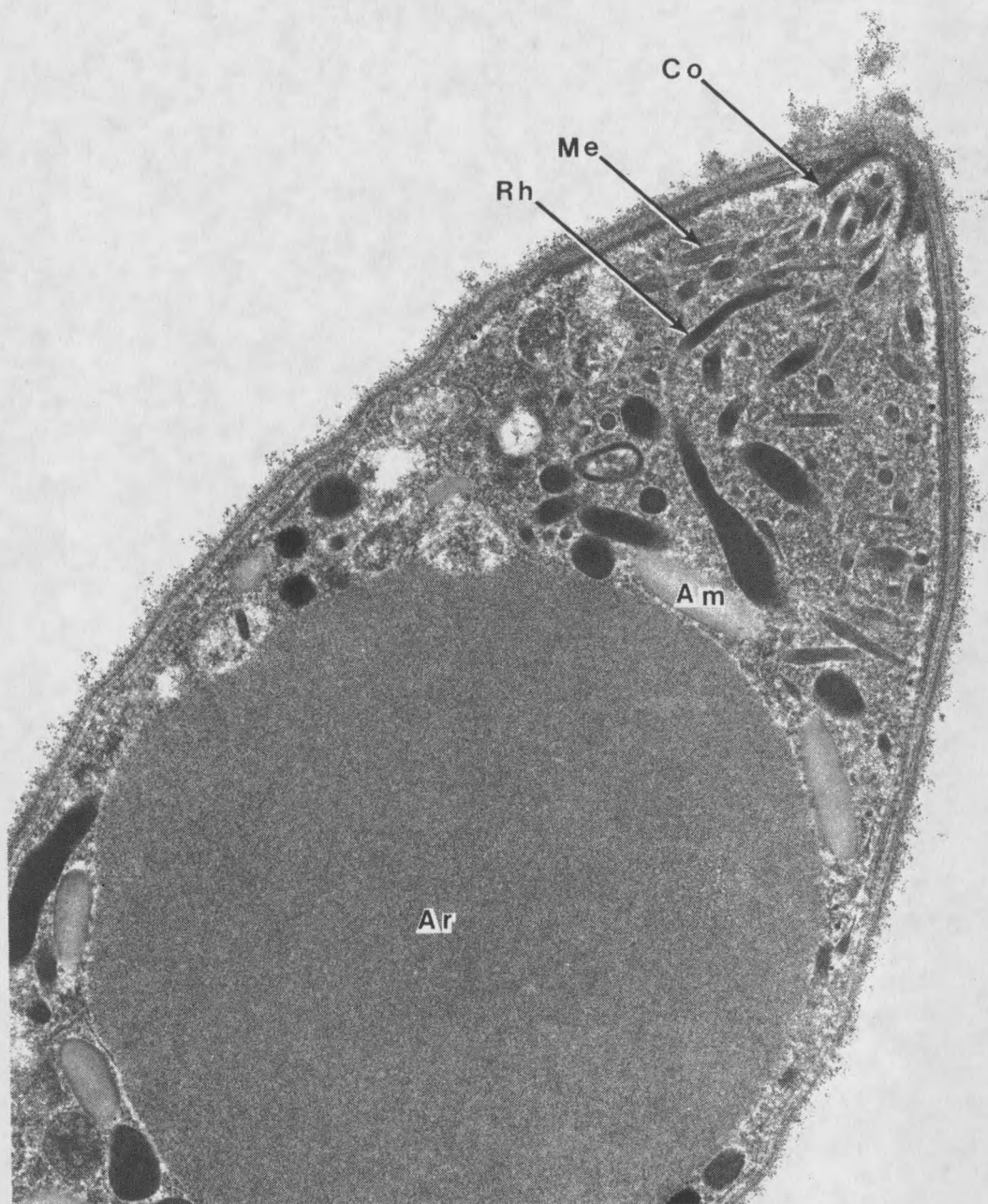


Fig. 20. Ultrastructural localization of parasite-specific IgG on the plasmalemma at the apical end of an *E. bovis* sporozoite. Note the ferritin-labelled membrane-bound vesicles at the anterior tip of the sporozoite. Am, amylopectin; Ar, anterior refractile body; Co, conoid; Me, microneme; Rh, rhoptry. Treatment: Prefixed, antiserum, ferritin-conjugated rabbit antiovine IgG. X 39,000.

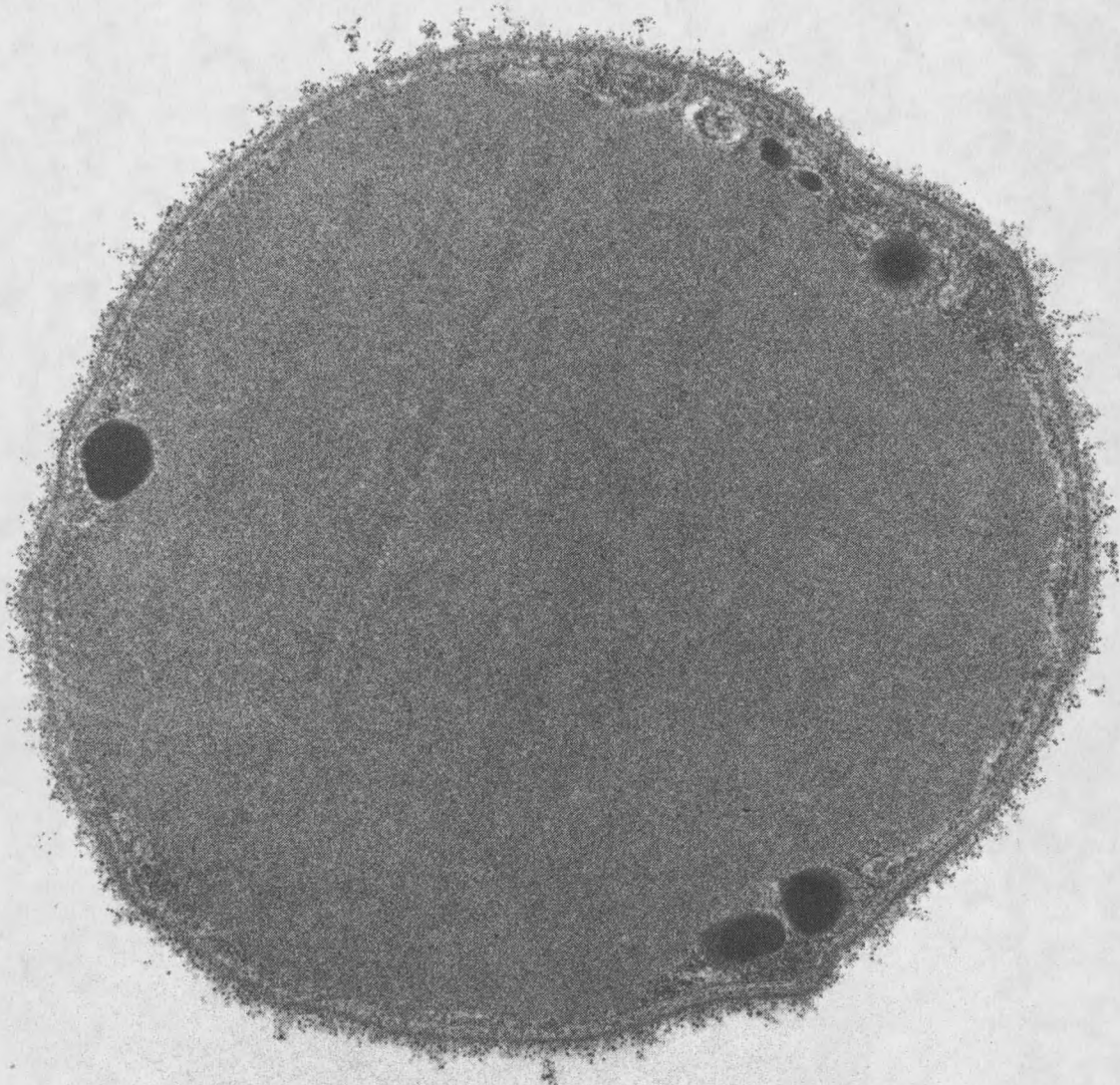


Fig. 21. Ultrastructural localization of parasite-specific IgG on the surface of a sporozoite. Cross-section of a sporozoite with a relatively uniform layer of ferritin on its surface. Treatment: Prefixed, antiserum, ferritin-conjugated rabbit antibovine IgG. X 48,000.

sporozoites, exposed to NS and then ferritin-conjugated antiovine IgG had little or no ferritin attached to their surfaces (Fig. 22).

Sporozoites treated with AS or IS for 5, 10 or 20 min before being fixed in glutaraldehyde capped immune complexes at their posterior ends (Figs. 23-25). These immune complexes were comprised of sporozoite antigen and sporozoite-specific bovine antibodies which were detected by ferritin-conjugated antiovine IgG antibody. Ultrastructurally, the capped immune complexes consisted of ferritin, fine granular material and membrane-bound vesicles which either remained attached to the sporozoite plasmalemma or were shed (Figs. 23-25). Sporozoites exposed to AS or IS for 10 or 20 min and then post-fixed had less ferritin on their plasmalemmae than did prefixed sporozoites (compare Figs. 20 and 23). The membrane-bound vesicles originated from blebs of the plasmalemma and sporozoite cytoplasm which evidently pinched off from the plasmalemma at the posterior of the sporozoite (Fig. 24). These vesicles then coalesced to form the cap of immune complexes. Some of the largest blebs of plasmalemma and cytoplasm occurred immediately above the posterior pore (Fig. 24). Although the cap appeared relatively large, no alterations were detected in the ultrastructural characteristics of the sporozoite other than loss of some plasmalemma and cytoplasm (Figs. 24, 25).

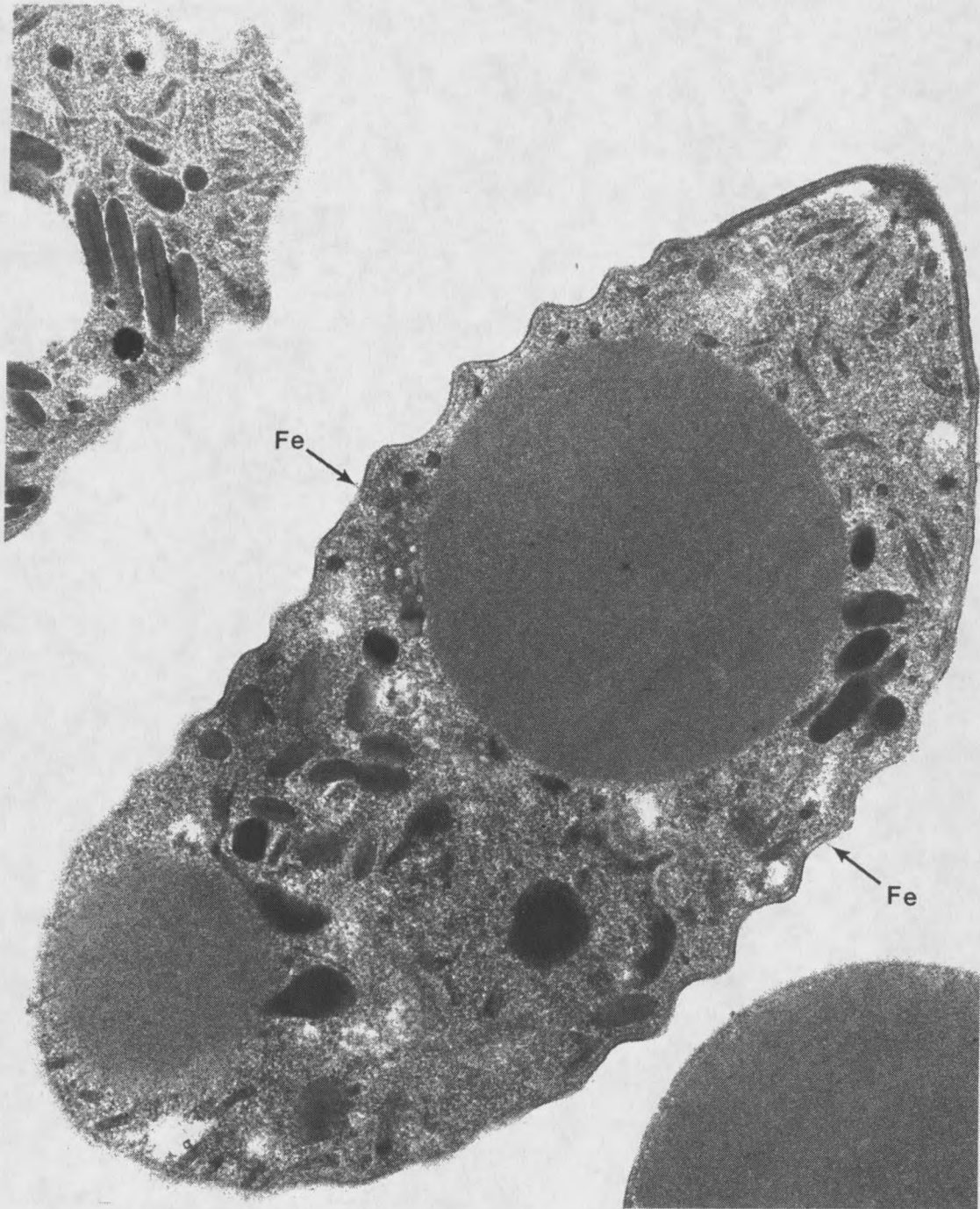


Fig. 22. Transmission electron micrograph of three prefixed *E. bovis* sporozoites which were exposed to normal serum followed by ferritin-conjugated rabbit anti-bovine IgG. Note almost complete absence of ferritin (Fe) on sporozoites. X 30,000.

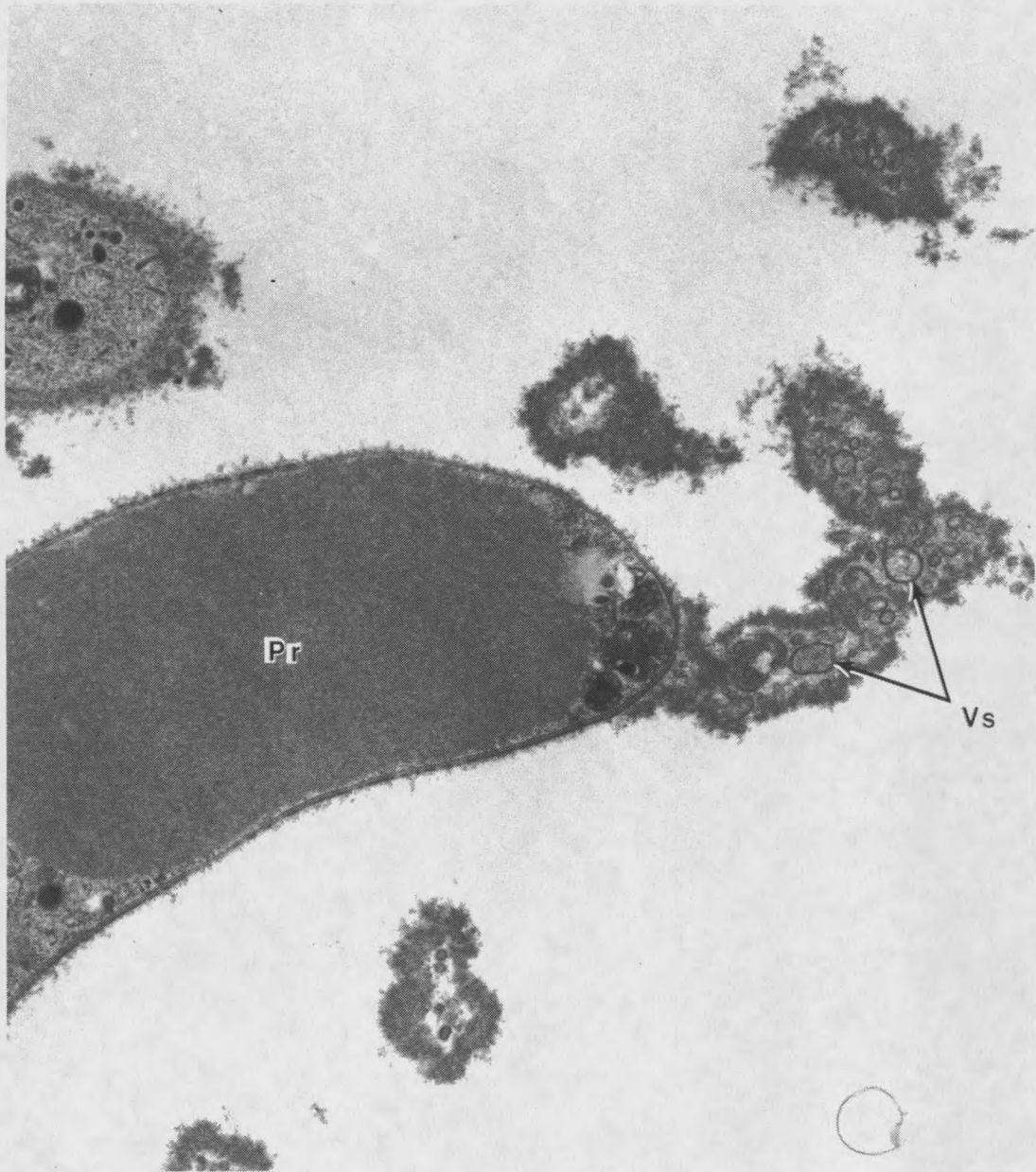


Fig. 23. Transmission electron micrograph showing capping of immune complexes at the posterior end of a sporozoite. The specimen was postfixed after a 10 min exposure to immune serum. Note that the capped immune complex contains fine granular material as well as membrane-bound vesicles (Vs). Pr, posterior refractile body. Treatment: Immune serum, postfixed, ferritin-conjugated rabbit antibovine IgG. X 15,000.

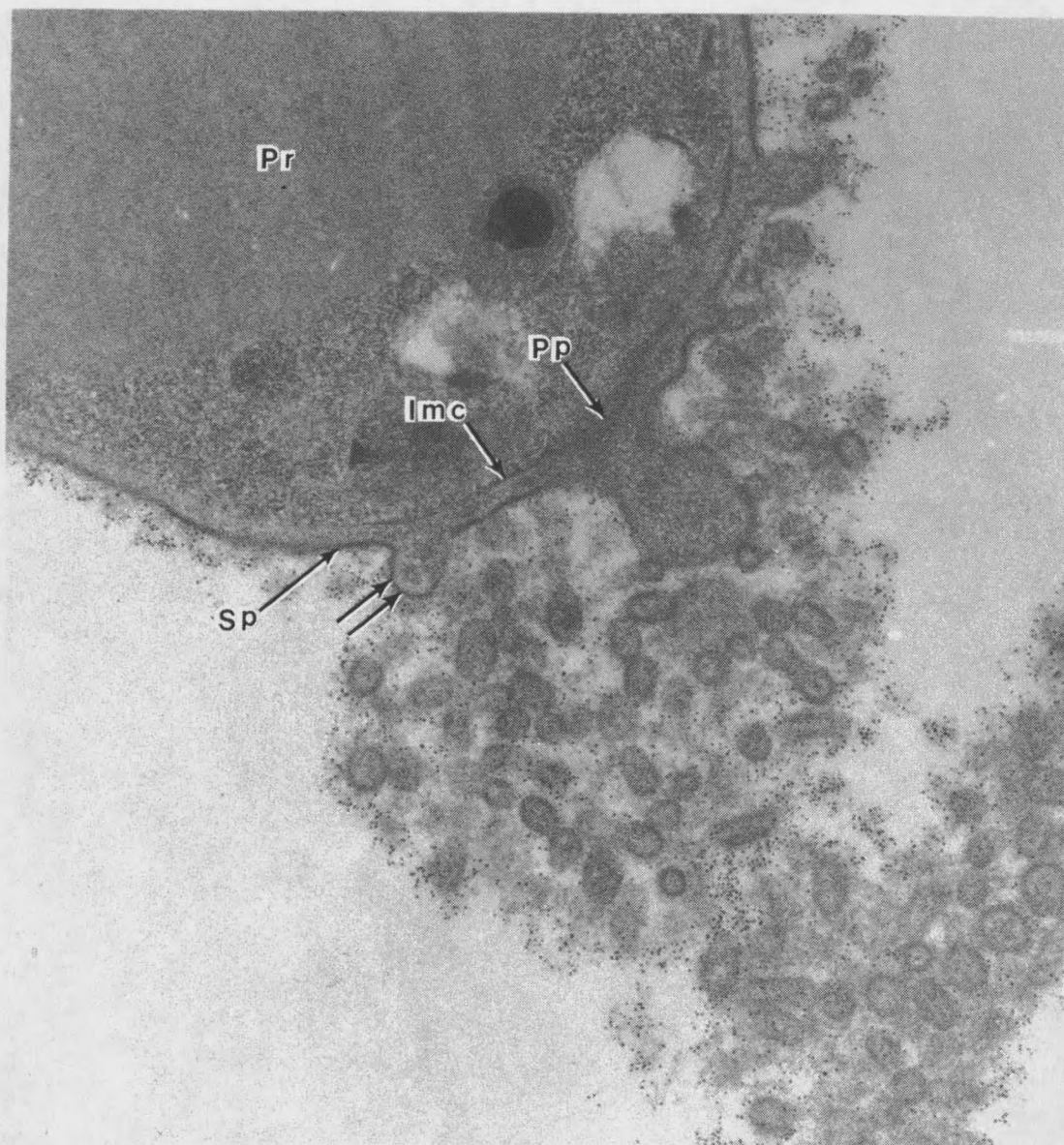


Fig. 24. High magnification transmission electron micrograph of the posterior end of a sporozoite showing capped immune complexes after a 10 min exposure to antiserum. Note blebs (double arrows) in the sporozoite plasmalemma (Sp) as well as membrane-bound vesicles in the capped immune complex; the bleb that developed over the area of the posterior pore (Pp) contains parasite cytoplasm. Imc, inner membrane complex; Pr, posterior refractile body. Treatment: Antiserum, postfixed, ferritin-conjugated rabbit antibovine IgG. X 60,000.

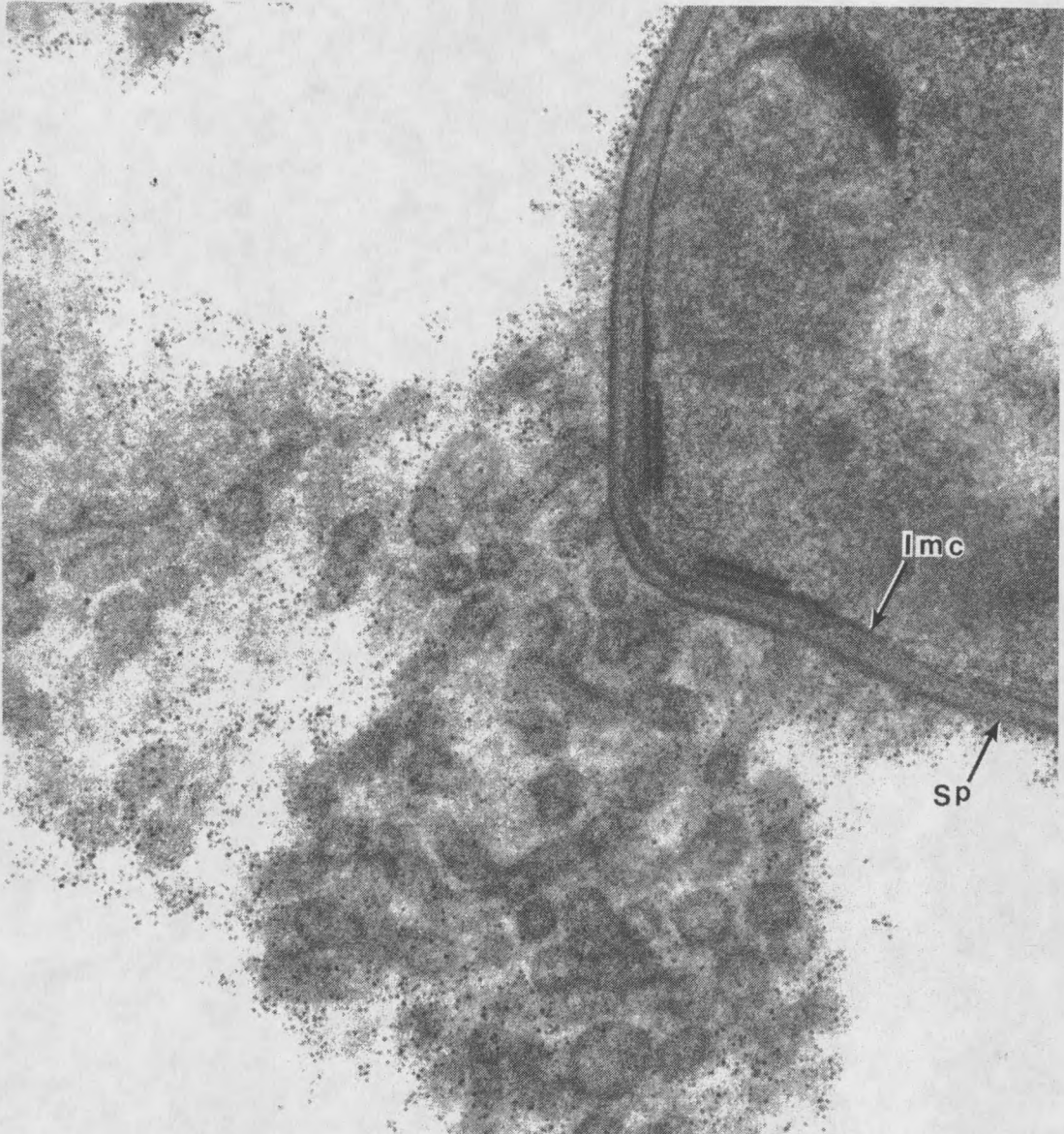


Fig. 25. High magnification transmission electron micrograph of the posterior end of a sporozoite after exposure to antiserum for 10 min. Note ferritin attached to sporozoite plasmalemma and membrane-bound vesicles in cap. Imc, inner membrane complex; Sp, sporozoite plasmalemma. Treatment: Antiserum, postfixed, ferritin-conjugated rabbit antiovine IgG. X 78,000.

Sporozoites that were prefixed in glutaraldehyde, exposed to EbS9 or EbS11 (IFA titers of 160) and then treated with ferritin-conjugated antimouse IgG had little ferritin on their surfaces. In contrast, those that were exposed to EbS9 or EbS11 and postfixed had small to moderate amounts of ferritin localized in patches on their plasmalemma (Figs. 26, 27). Unlike sporozoites that had been postfixed following exposure to AS or IS, sporozoites exposed to EbS9 and then postfixed did not cap nor shed (compare Figs. 23 and 26). No ultrastructural alterations were detected in sporozoites exposed to EbS9 or EbS11. No ferritin occurred on the surfaces of sporozoites exposed to Ag8, postfixed and treated with ferritin-conjugated antimouse IgG (Fig. 27).

#### Sporozoite Antigen Analysis

SDS-PAGE (12.5% polyacrylamide) of E. bovis sporozoites stained with Coomassie brilliant blue revealed a profile of proteins ranging in  $M_r$  from approximately 15,000 to more than 200,000 (Fig. 28). Several major protein bands of  $M_r$  less than 68,000 were clearly resolved (Fig. 28).

Western blot analysis of sporozoite proteins revealed that both EbS9 and EbS11 reacted with a protein(s) of approximately 20,000  $M_r$  (P20) (Fig. 28). Western blots of sporozoite proteins probed with Ag8 indicated that immunodetection of other higher  $M_r$  proteins in lane C and D of

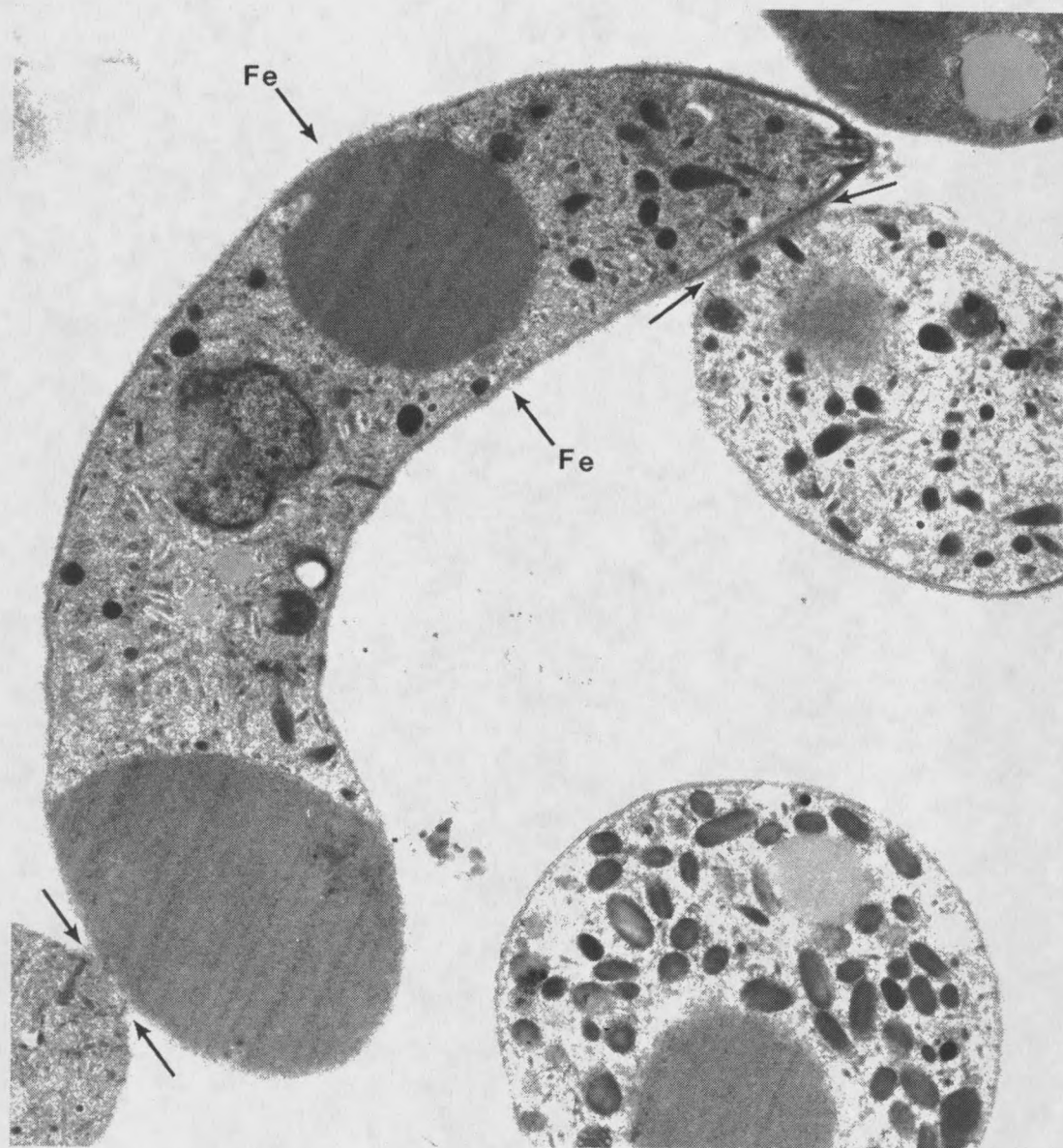


Fig. 26. Ultrastructural localization of ferritin (Fe) on the surfaces of *E. bovis* sporozoites after 10 min exposure to EbS11. Note the ferritin is not as dense as with antiserum or immune serum (Figs. 20 and 23, respectively), the slight agglutination (arrows) and the absence of capping of immune complexes. The sporozoite in the lower right is probably another *Eimeria* spp. since little or no ferritin is present on its surface. Treatment: EbS11, postfixed, ferritin-conjugated rabbit antimouse IgG. X 15,000.

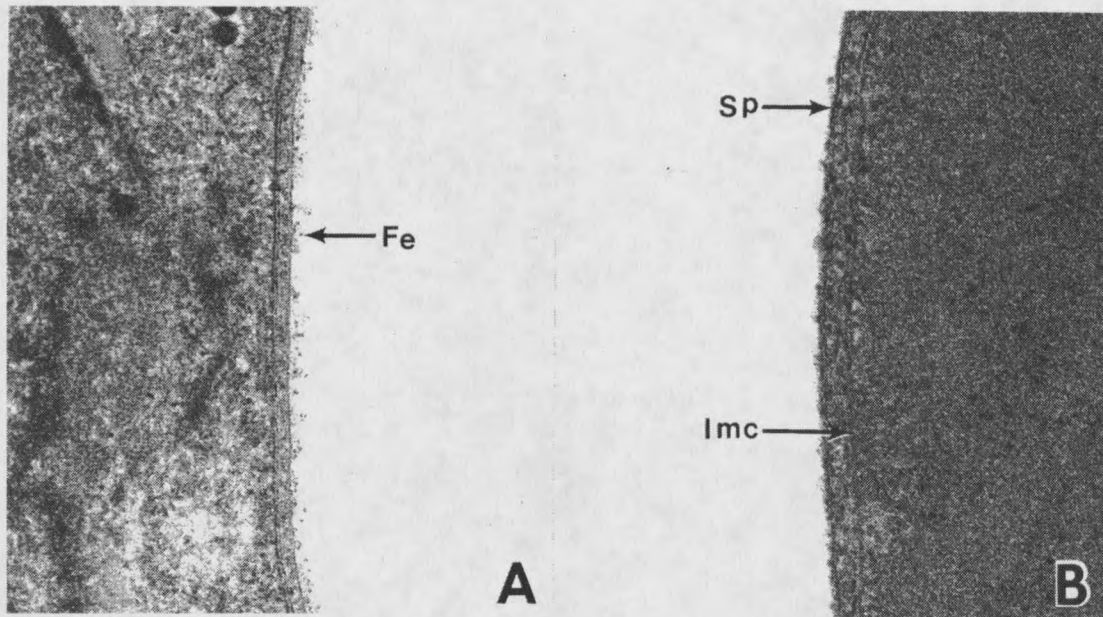


Fig. 27. A. Ultrastructural localization of ferritin on the surface of *E. bovis* sporozoite after exposure to EbS9 for 10 min. Note thin but uniform layer of ferritin (Fe). Treatment: EbS9, postfixed, ferritin-conjugated rabbit antimouse IgG. X 60,000. B. High magnification of the pellicle of an *E. bovis* sporozoite following exposure to Ag8 for 10 min. Note the lack of ferritin on the plasmalemma (Sp). Imc, inner membrane complex. Treatment: Ag8, postfixed, ferritin-conjugated rabbit antimouse IgG. X 99,000.

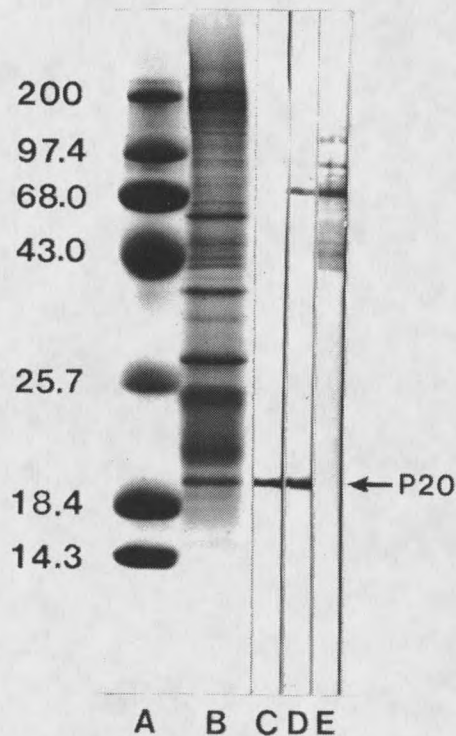


Fig. 28. SDS-PAGE (12.5% acrylamide) showing a protein profile of nonreduced *E. bovis* sporozoites (lane B) stained with Coomassie brilliant blue, and western blots of similar sporozoite proteins. The western blots were probed with EbS9 (lane C), EbS11 (lane D) and Ag8 (lane E). The arrow indicates the  $M_r$  20,000 (P20) sporozoite protein band against which EbS9 and EbS11 react. The higher  $M_r$  protein bands that are visible in all three western blots are probably due to nonspecific cross-reactivity of serum proteins in the concentrated CM. Lane A consists of prestained BRL molecular weight standards ( $\times 10^3$ ).

Fig. 28 was nonspecific. However, in western blots of sporozoite proteins run under reduced conditions (i.e. in the presence of 2-mercaptoethanol), EbS9 and EbS11 did not react with P20 or any apparent subunit of this antigen (Fig. 29).

Autoradiographic analysis of radioiodinated sporozoites revealed that sporozoites contained at least six surface proteins, one of which was identified as P20 by  $M_r$  calculations (Fig. 30). The relative positions of the 6 distinct sporozoite-surface proteins that were identified by autoradiography are displayed by a Coomassie brilliant blue stained 12.5% polyacrylamide gel in Fig. 31. However, since the linear relationship of the BRL prestained molecular weight standards was maintained only for molecular weights of 14,500 to 68,000, the relative position of the first sporozoite-surface protein (protein 1) on Coomassie brilliant blue stained gels was assigned by its position relative to the prestained standards (Fig. 31). Since the  $M_r$  of this sporozoite-surface protein could not be determined from a standard curve, it was not included in the following sporozoite antigen analysis. The other five sporozoite-surface proteins had the following  $M_r$ : Protein 2, 54,900; 3, 42,700; 4, 40,700; 5, 25,000; and P20, 20,000 (Fig. 31). Nonreduced sporozoite-surface proteins 2, 4, 5 and P20 appear to be major sporozoite proteins in Coomassie brilliant blue stained 12.5% polyacrylamide gels (Fig. 31).

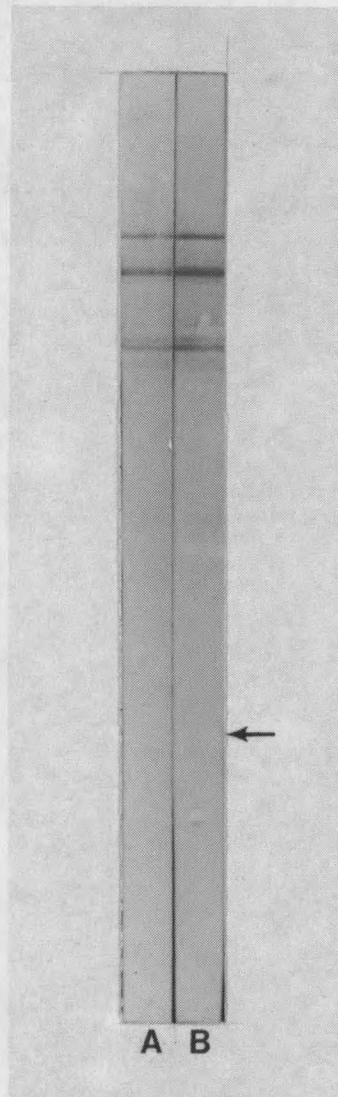


Fig. 29. Western blot analysis of reduced *E. bovis* sporozoite proteins that were transferred from a 12.5% SDS-polyacrylamide gel to nitrocellulose paper. EbS9, lane A; EbS11, lane B. Note that EbS9 and EbS11 did not bind in the  $M_r$  20,000 region (arrow) of the blots, indicating that the epitopes normally recognized by these MAbs were destroyed under reducing conditions. The high  $M_r$  bands that are visible in the western blots are probably due to cross-reactivity of serum proteins in the concentrated CM.

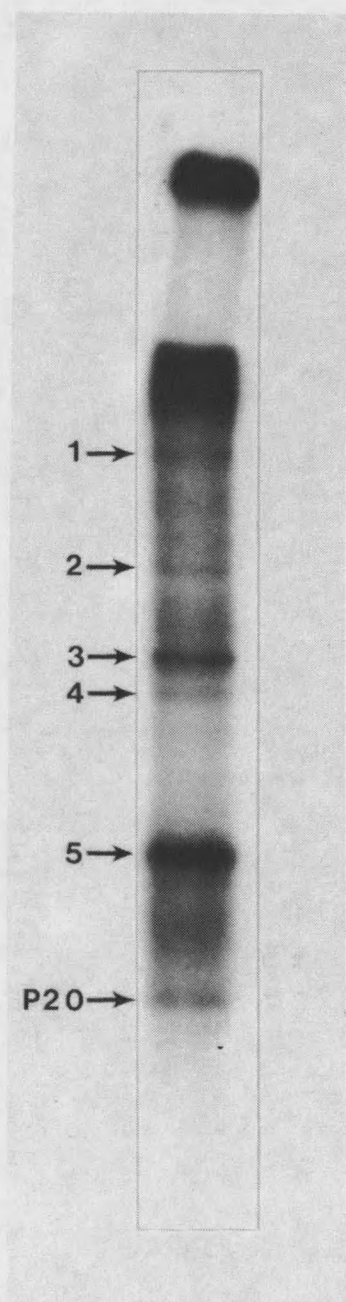


Fig. 30. Autoradiographic profile of  $^{125}\text{I}$ -labelled surface proteins of nonreduced *E. bovis* sporozoites (12.5% SDS-polyacrylamide gel). The arrows indicate the relative positions of six labelled protein bands, one of which correlates to  $M_r$  20,000 (P20).

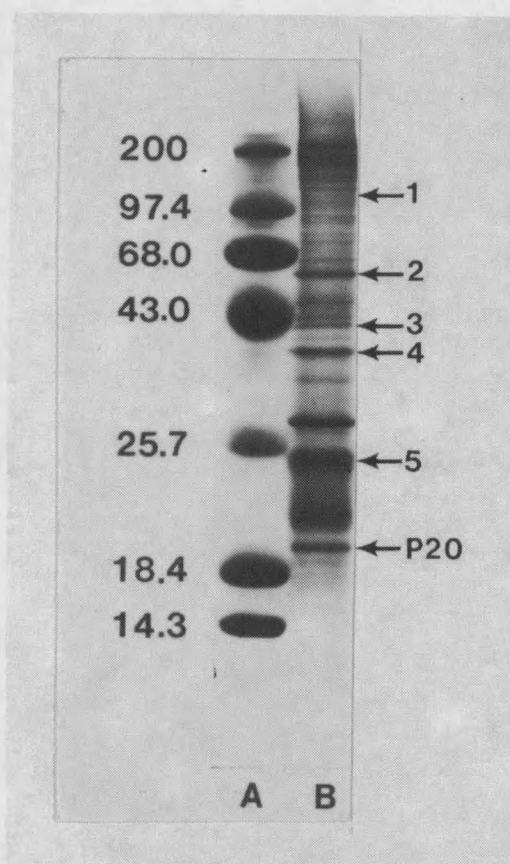


Fig. 31. SDS-PAGE of nonreduced *E. bovis* sporozoites (lane B) stained with Coomassie brilliant blue. The arrows indicate the relative positions of six protein bands that correspond to those proteins identified by autoradiography (Fig. 30). P20 indicates the  $M_r$  20,000 sporozoite surface protein against which EbS9 and EbS11 react. Lane A consists of BRL prestained molecular weight standards ( $\times 10^3$ ).

When sporozoites were solubilized under reducing conditions, only protein 4 could be identified in SDS-PAGE (stained with Coomassie brilliant blue), indicating that the other three proteins contain disulfide bonds (data not shown).

Several sporozoite proteins reacted with AS and IS on western blots of nonreduced sporozoite proteins (Fig. 32). AS detected 5 sporozoite proteins that correlated with sporozoite-surface proteins that were identified by autoradiography (Fig. 32). However, IS reacted with only 4 of the 5 sporozoite-surface proteins (proteins 2, 3, 4 and P20) (Fig. 32). Both AS and IS appeared to react intensely with nonreduced P20 (Fig. 32). NS did not react with western blots of nonreduced sporozoite proteins (Fig. 32).

#### Immunodetection of Antigens During Parasite Development

At 1 to 8 and 12 days ASI (the days ASI tested) of MDBK cells, EbS9 reacted by IFA with intracellular E. bovis sporozoites but not with first-generation meronts that had been prefixed with acetone (Figs. 33, 34). EbS9 reacted similarly in IFA assays with sporozoites in M617 cells. On day 12 ASI, however, EbS9 reacted intensely with a granular material within the parasitophorous vacuole of M617 cells surrounding intermediate meronts and a moderate IFA reaction occurred within the meront (Fig. 35). No fluorescence was seen in the parasitophorous vacuole surrounding meronts in

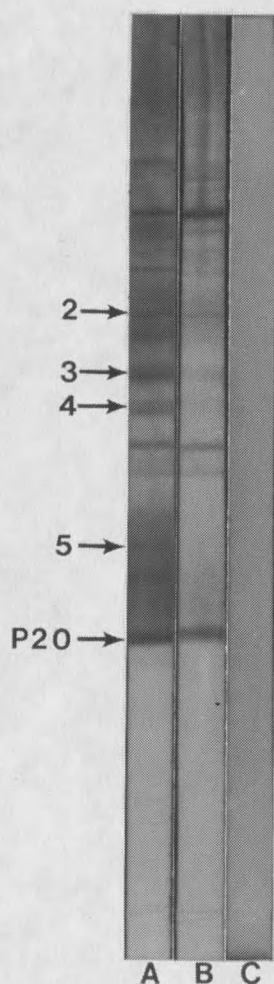


Fig. 32. Immunodetection of nonreduced *E. bovis* sporozoite antigens by antiserum (lane A) and immune serum (lane B) on western blots (12.5% SDS-polyacrylamide gel). The arrows indicate the relative positions of five sporozoite surface proteins that were identified by autoradiography (Fig. 30). P20 indicates the  $M_r$  20,000 sporozoite surface protein against which EbS9 and EbS11 react (Fig. 28). Lane C represents a western blot that was probed with normal serum.

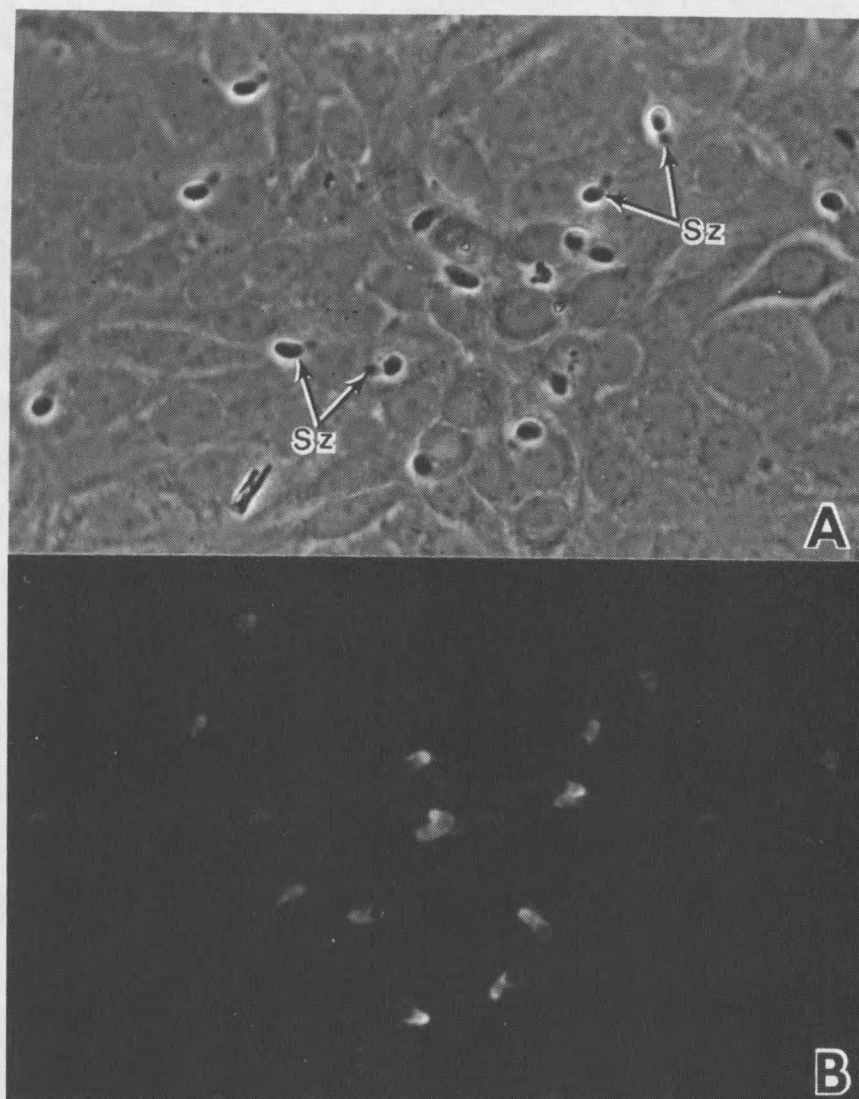


Fig. 33. Photomicrographs of *E. bovis* sporozoites in MDBK cells. A. Phase-contrast photomicrograph of several intracellular sporozoites (Sz) 24 hr ASI. X 400. B. IFA of the same specimens in A after exposure to EbS9 which has reacted with the sporozoites. Treatment: Prefixed, EbS9, fluorescein-conjugated goat antimouse IgG. X 400.

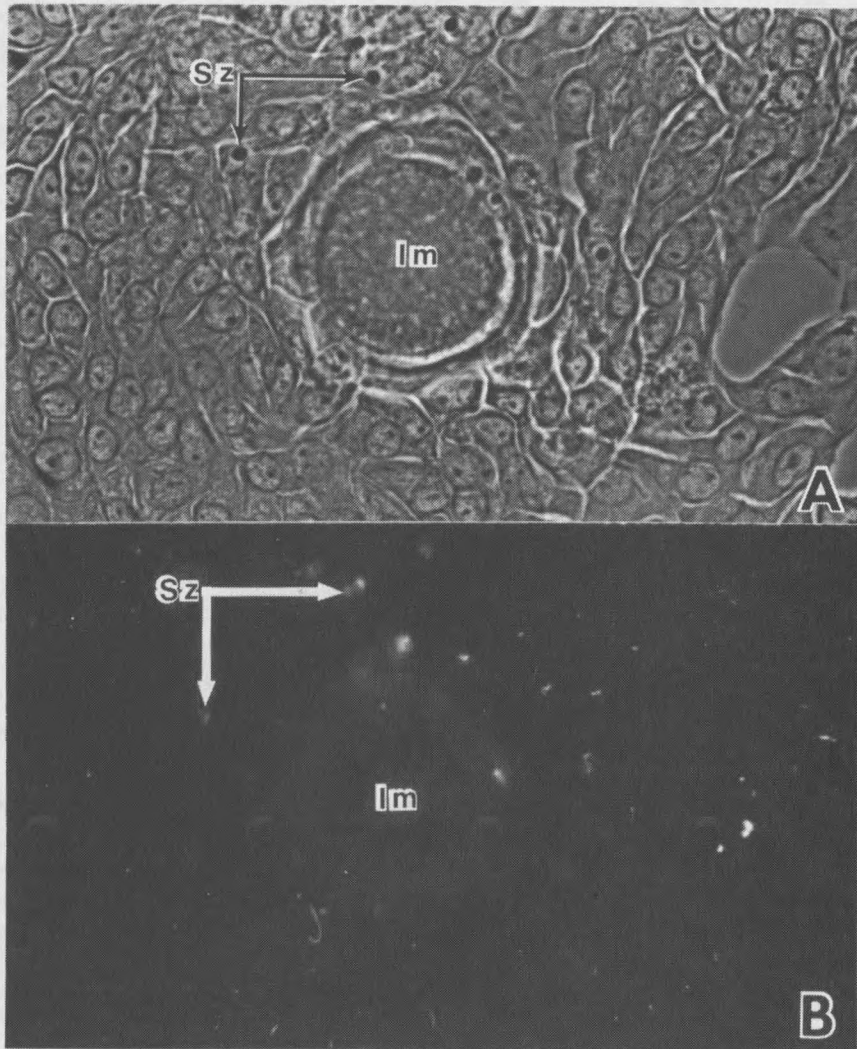


Fig. 34. Phase-contrast photomicrograph of several *E. bovis* sporozoites (Sz) and an intermediate meront (Im) in MDBK cells 12 days ASI. X 200. B. IFA of the same specimens as A after exposure to EbS9. Note the positive fluorescence of the intracellular sporozoites (Sz) as well as the negative reaction of the intracellular meront (Im). Treatment: Prefixed, EbS9, fluorescein-conjugated goat anti-mouse IgG. X 200.

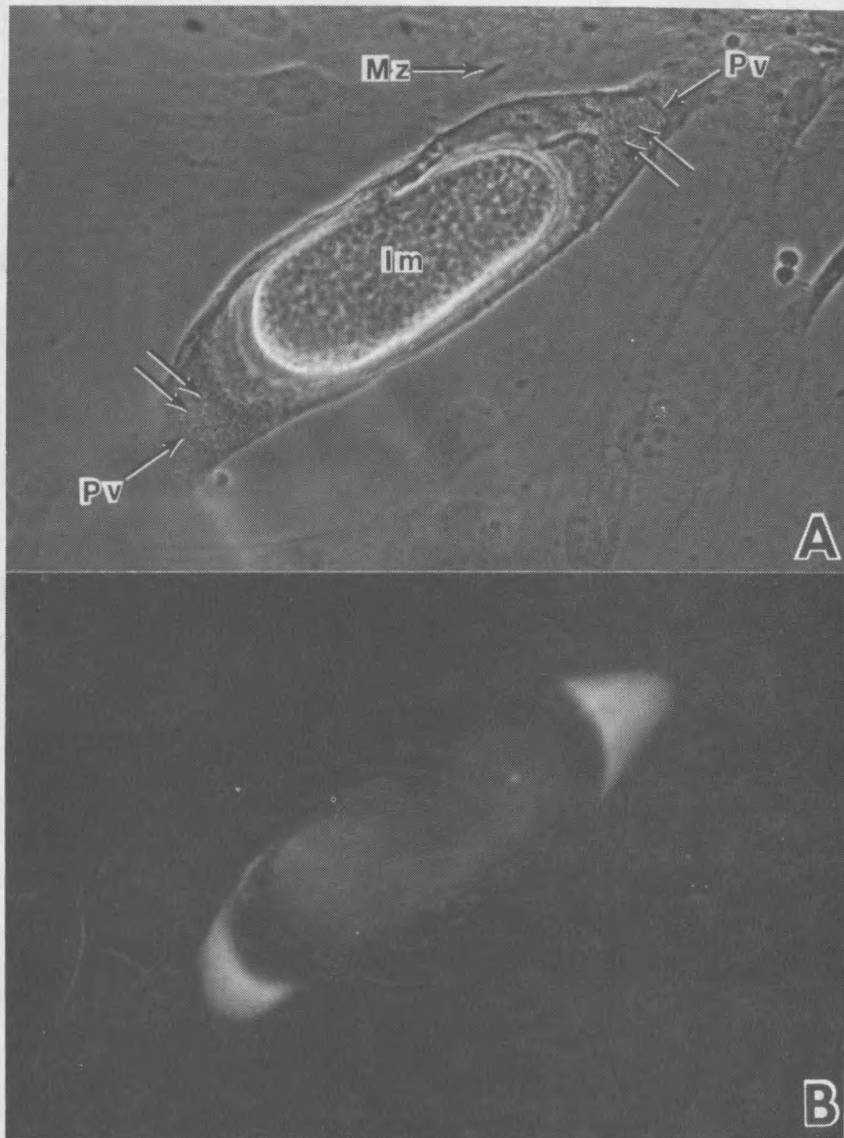


Fig. 35. Phase-contrast photomicrograph of an intermediate meront (Im) of *E. bovis* in M617 cells 12 days ASI. Note the granular material (double arrows) between the meront and the parasitophorous vacuolar membrane (Pv). Mz, merozoite. X 200. B. IFA of the same specimens as A after exposure to EbS9. Note that the material in the parasitophorous vacuole fluoresces intensely but the extracellular merozoite does not fluoresce. Treatment: Pre-fixed, EbS9, fluorescein-conjugated goat antimouse IgG. X 200.

MDBK cells treated with EbS9 (Fig 34). In cultures of M617 cells, prefixed intracellular and extracellular first-generation merozoites did not exhibit fluorescence after exposure to EbS9 and then fluorescein-conjugated antimouse IgG antibody (Fig. 35). EbS9 did not react with MDBK or M617 cells during the IFA assay (Figs. 34, 35).

## DISCUSSION

All members of the Phylum Apicomplexa are obligate intracellular parasites. Except for the process of sporogony in certain groups (i.e., Coccidia), all apicomplexans must be intracellular in order to undergo asexual and sexual reproduction. Although the process of penetration of cells by coccidian parasites has been the subject of several reports, it is still poorly understood. Apicomplexans gain entry into cells by using their apical complex in some unknown way to actively penetrate through the plasmalemma of the host cell. Essentially nothing is known regarding the roles of components of the apical complex, enzymes, receptors or other factors. Toxoplasma gondii will penetrate macrophages and cause disruption of the macrophage plasmalemma (45). Eimeria magna sporozoites will penetrate MDBK cells, in which phagocytosis was chemically suppressed (74). Direct microscopical examination indicates that sporozoites, merozoites or tachyzoites actively penetrate their host cells and do not rely on the host cell to phagocytose them (85, 110, 111, 114). Thus, it appears that coccidian parasites penetrate without being phagocytosed by the host cell. However, E. magna, I. canis, Plasmodium spp. and B. microti apparently do not disrupt the host cell plasmalemma during penetration, but cause an

invagination of the host cell plasmalemma until the parasites are entirely within the cell (44, 46, 47, 57, 105). Sporozoites of E. bovis also do not appear to disrupt the host cell plasmalemma during penetration. This finding may partially account for observations during earlier investigations (85, 114) as well as in the present study, that host cell cytoplasm was rarely seen to escape during penetration.

In the case of E. bovis, however, the indented portion of the host cell plasmalemma apparently surrounds the intracellular sporozoites only momentarily, since sporozoites that were completely intracellular after 5 or 10 min were either free in the host cell cytoplasm or only partially surrounded by short segments of the host cell plasmalemma. These results corroborate those of Jensen and Hammond (44) who reported similar findings with E. magna sporozoites within MDBK cells. Moreover, in the present study, the relatively short existence of the invaginated host cell membrane is underscored by observations of E. bovis sporozoites that were in the process of exiting from host cells. At the site of exit, the sporozoite was enveloped completely by host cell plasmalemma and cytoplasm yet there was no evidence of the original invaginated plasmalemma in close proximity to the sporozoite. Thus, although the host cell plasmalemma initially surrounds penetrating sporozoites of E. bovis the membrane quickly

disappears and is eventually replaced by a new membrane (the parasitophorous vacuolar membrane) which likely originates from the host cell endoplasmic reticulum. Jensen and Hammond (44) observed a similar sequence of events and proposed that the parasitophorous vacuolar membrane surrounding sporozoites of E. magna was of host cell origin.

Although there is substantial evidence that suggests immunity to coccidiosis is primarily T-cell dependent, appreciable amounts of parasite-specific immunoglobulins are produced by infected hosts (1, 16, 72, 86, 93, 95, 99, 126). It has been suggested that the humoral response plays a role in the modulation of primary infections either as a first line of defense or as a modifier of the cellular response (100).

In the present study, the patency period through which E. bovis oocysts were shed during primary infection corresponded with the rise and fall of the IgG titers in the infected animals. For example, on day 14 after the initial oral inoculation of each of 2 calves with oocysts of E. bovis, parasite-specific IgG IFA titers of 20 were demonstrable in the serum of both animals. Both calves passed oocysts at 18 to 31 days after inoculation. Peak combined oocyst discharge ( $185 \times 10^6$  oocysts) occurred at 19 days after inoculation which was coincidental with the peak parasite-specific IgG serum titer of 40. Four days after

patency (i.e., 35 days after oocyst inoculation), serum antibody titers returned to pre-inoculation levels of 10.

These findings are complimentary to those of Andersen et al. (1) in that the average peak oocyst discharge occurred at 19.1 days after an initial oral inoculum of E. bovis oocysts. They also reported an average peak IFA parasite-specific humoral response (titers of approximately 80) at 21 days (1). However, these investigators incorporated first-generation merozoites as the antigen for IFA assays and did not detect fluorescence until 10 to 22 days (1). This seems logical since this merogonous stage of the parasite does not reach maturity until about 14 or 15 days after inoculation of oocysts (38). In contrast, sporozoites of E. bovis were used here as the IFA antigen and immunofluoresence was detected by 7 days after the initial inoculation. Parasite-specific IgG against first-generation merozoites was observed at 21 days after inoculation as well.

Rose et al. (100) reported that different subclasses of parasite-specific serum IgG reached peak levels between 20 to 30 days after oocyst inoculation of rats with E. nieschulzi oocysts. These animals also demonstrated an anamnestic IgG antibody response to a challenge inoculum of oocysts (100). However, Andersen et al. (1) were unable to demonstrate a clear secondary humoral response in calves challenged with oocysts of E. bovis at 26 to 27 days after

the primary oocyst inoculation. These authors suggested that longer intervals between inoculations may be necessary in order to demonstrate an optimum secondary humoral response. ✓ Investigators using other animal-parasite systems have reported that certain eimerians fail to stimulate a detectable secondary humoral response. For example, Rose (87) found that rabbits challenged with oocysts of E. stiedai after recovery from near-fatal infections did not demonstrate increases in antibody titers by complement-fixation or gel-diffusion tests. ✓

The anamnestic humoral response to E. bovis (present study) was clearly evident 6 weeks after the initial inoculation. The straight-line appearance of the secondary response during the last 7 weeks was probably due to the use of 2-fold dilutions of IS for this experiment. During this time, the IFA titers were always less than 320 but always detectable at a titer of 160. Although these serum anti-E. bovis IgG titers do not appear to be particularly high, they are within the titer range of 150 to 640 that was reported by Andersen et al. (1) for calves receiving two inoculations of E. bovis oocysts.

An oral inoculation of  $5 \times 10^4$  oocysts of the strain of E. bovis used in our laboratory has proven to be a lethal dose to previously noninfected bovine calves. No clinical signs of coccidiosis were exhibited by the calves after a challenge inoculation of  $5 \times 10^4$  oocysts and only a single

oocyst per gram of wet feces was discharged by each calf on what normally would have been within the interval of peak oocyst discharge during primary infection. This result agrees with several investigators who have concluded that immunity to Eimeria spp. does not prolong or delay the rate of parasite development, but does significantly reduce the numbers of parasites reaching maturity in immunized calves (37, 39).

In the present study, all of the MAbs were elicited against the sporozoite stage of E. bovis and demonstrated at least 4 different fluorescent binding patterns on acetone-fixed sporozoites by IFA assays. This clearly indicates that the various MAbs are directed against several different sporozoite antigens. Similar results were reported earlier by Danforth (14) who described hybridoma-secreted antibodies which produced 8 different IFA patterns on air-dried E. tenella and E. mitis sporozoites. All of these antibodies were species-specific and did not cross-react by IFA with any of several eimerians of poultry (14).

In the present study, 3 MAbs (EbS7, 15 and 16) also reacted against acetone-fixed in vitro-produced first-generation merozoites of E. bovis. This finding is not so surprising since these merozoites develop from sporozoites and might be expected to share some common antigens. Similarly, Kasper et al. (52) detected shared antigens between T. gondii sporozoites and tachyzoites with

polyclonal as well as MAbs. Moreover, one of the 3 E. bovis MAbs described above, EbS16, also reacted with acetone-fixed sporozoites of another bovine eimerian tentatively indentified as E. ellipsoidalis or E. zuernii. This finding indicates that certain antigens may be shared among different bovine Eimeria species. In support of this, other investigators have recently found a MAb that reacts with the surface of 4 Eimeria spp. that infect poultry (4). However, in the present study, EbS7, 15 and 16 did not react with live IFA preparations of E. bovis sporozoites or merozoites, which indicates that these shared antigens are not exposed on the surfaces of either stage of the parasite. All of the other MAbs proved to be sporozoite-specific and only 2 of these, EbS9 and EbS11, respectively, reacted against sporozoite surface antigens by live IFA assays.

Sporozoite agglutination occurred during live IFA assays with EbS9 and EbS11, indicating that the antigens against which these MAbs react are located on the sporozoite surface. EbS9 and EbS11 also reacted with the inner surface of the sporocyst wall, especially that portion near the Stieda body, which indicates that different parasite structures of E. bovis share common antigens.

It was interesting to note that EbS9 and EbS11 produced a characteristic strong apical region fluorescence pattern with acetone-fixed sporozoites and a diffuse whole body fluorescence with live sporozoites. The whole body

fluorescence produced by live IFA techniques was possible because EbS9 and EbS11 had reacted against surface antigens of living sporozoites before fixation in glutaraldehyde, which stabilized the plasmalemmae of the sporozoites. However, since acetone is a lipid solvent, sporozoites that were prefixed in acetone before exposure to MAbs would have lost the integrity of their plasmalemmae and inner membrane complex. This condition provided the MAbs with access to the sporozoite interior. Thus, the apical fluorescence exhibited by acetone-fixed sporozoites exposed to EbS9 or EbS11, indicates that these MAbs also react with internal sporozoite antigens. The weak, patchy fluorescence that was seen with most of the sporozoites in conjunction with the strong apical region fluorescence probably represents small amounts of sporozoite plasmalemma that retained EbS9 and EbS11-specific antigens after acetone-fixation.

Although both EbS9 and EbS11 appear to react with only a single sporozoite protein band on western blots, it is possible that these MAbs may have reacted by IFA to intracellular surface-antigen precursors within acetone-fixed sporozoites. Recently, the distribution of a protective surface antigen of P. knowlesi sporozoites as well as the intracellular localization of its precursors have been determined by cryoultramicrotomy in conjunction with surface antigen-specific MAbs and colloidal gold-conjugated Protein A (29). The surface antigen precursors were found to be

associated with micronemes and rhoptries in the apical region of the parasite (29). This indicates that micronemes and rhoptries may serve to store and transport the polypeptide to the anterior tip of the sporozoite where it is secreted or inserted into the plasmalemma of the sporozoite. Although I did not determine if the antigen against which EbS9 and EbS11 react was secreted, live IFA indicated that it was inserted into the sporozoite plasmalemma. A penetration enhancement factor that enhances the entry of T. gondii into mammalian cells has been recently shown to be associated with T. gondii rhoptries by MAbs (109).

Similar scenarios for the involvement of micronemes and rhoptries in cell penetration are likely to exist for E. bovis sporozoites as well as for zoites of most other Apicomplexans. However, ultrastructural studies such as those described above concerning P. knowlesi and T. gondii need to be performed to determine which organelles may be responsible for the apical fluorescence displayed by sporozoites of E. bovis following exposure to EbS9 or EbS11.

Additional IFA studies with EbS9 demonstrated that differences existed between first-generation meronts in MDBK and M617 cells. EbS9 reacted intensely with granular material located within the parasitophorous vacuole surrounding meronts of E. bovis in M617 but not MDBK cells

indicating differences in the fate or processing of P20 in these cells. As meront development progressed, their ability to fluoresce when treated with EbS9 or EbS11 gradually diminished and merozoites exhibited negative fluorescence. The fluorescent granular material within the parasitophorous vacuole of M617 cells may represent sequestered P20 antigen that is shed during parasite development. Conversely, the lack of fluorescence by meronts or the parasitophorous vacuole in MDBK cells indicates that P20 is shed but not sequestered by these cells. Also, EbS9 does not react with the parasitophorous vacuolar membrane in either cell line, indicating that this membrane is probably derived from the host cell and not the sporozoite.

Both EbS9 (IgG<sub>1</sub>) and EbS11 (IgG<sub>2a</sub>) reacted with the same E. bovis sporozoite protein band (P20) on western blots of nonreduced sporozoites. However, EbS9 and EbS11 did not react on western blots of reduced sporozoite proteins with P20 or any reduced subunit thereof. This indicates that the antigenic determinants (epitopes) against which these 2 MAbs react are dependent on the tertiary structure of sporozoite polypeptides (119). The high M<sub>r</sub> bands that reacted in western blots of both nonreduced and reduced sporozoites were probably due to serum proteins which were present in the HS that was used to fortify hybridoma CM. Evidence for this nonspecific immunodetection resides in the fact that

similar high  $M_r$  bands were detected in western blots that were probed with concentrated Ag8 which incorporated HS but did not contain murine MAbs. Additionally, when western blots of nonreduced sporozoites were probed with concentrated CM from EbS9, EbS11 or Ag8 cell cultures in which FBS was present, cross-reactivity to the high  $M_r$  bands was abolished and the only demonstrable reactivity was produced by EbS9 and EbS11 against P20 (data not shown).

Since EbS9 and EbS11 were isotypized as 2 unique IgG subclasses and only a single reactive band was observed on nonreduced western blots, these antibodies appear to be of true monoclonal distinction. Therefore, because there was no evidence of reactivity to other nonreduced transferred sporozoite proteins by these MAbs, it appears that no common amino acid sequences or repeated EbS9- or EbS11-reactive antigenic determinants are present in these proteins (6). Whether the P20 polypeptide contains different, identical or more than 1 epitope for EbS9 and EbS11 remains to be determined. Several researchers have shown that surface circumsporozoite proteins of various species of Plasmodium contain a single immunodominant region with two or more identical epitopes (130).

Autoradiography of nonreduced radiolabelled sporozoites in 12.5% polyacrylamide gels revealed the presence of 6 iodinated proteins, 1 of which corresponded to P20. This finding confirms the surface location of P20 by the

live IFA assays with sporozoites and EbS9 and EbS11. Additionally, 3 other iodinated proteins (proteins 2, 4 and 5) appear to correlate with major protein bands on Coomassie brilliant blue stained SDS-PAGE.

In contrast, Reduker and Speer (83) reported only 3 major iodlatable proteins of reduced E. bovis sporozoites in 12.5% polyacrylamide gels. Coomassie brilliant blue stained SDS-PAGE of reduced sporozoites in the present study revealed that protein 4 ( $M_r$  40,700) was the only iodlatable polypeptide that did not undergo reduction (data not shown). Yet, none of the  $M_r$  of the iodlatable proteins reported herein corresponded to those of the earlier investigation by Reduker and Speer (83). This discrepancy is difficult to explain since the strain of E. bovis used by these investigators was identical to the strain incorporated in the present study.

Western blots of nonreduced E. bovis sporozoites that were probed with AS or IS demonstrated the inherent immunogenicity of P20. Both AS and IS reacted intensely with P20, whereas AS detected at least 4 of the other 5 iodlatable protein bands (proteins 2-5) with varying intensities (Fig. 32). In addition to P20, IS reacted weakly with 3 other iodlatable protein bands (proteins 2, 3 and 4). A high  $M_r$  band that was located above protein 2 on western blots also reacted intensely with IS (as well as AS) but did not appear to correspond to a similar iodlatable band by

autoradiography. The differences between the ability of AS and IS to react with these proteins was probably due to the differences in the methods of immunization as well as antigen processing and presentation by host animals. Similarly, Reduker and Speer (83) found that immune bovine serum that was generated by different regimens varied in their immunoblotting profiles with reduced E. bovis sporozoite and merozoite antigens.

Since the IS used in the present study was obtained from an animal that survived an experimental E. bovis infection induced by oral inoculation of oocysts, the IS results on western blots were considered to more accurately reflect the actual immunogenic profile of nonreduced E. bovis sporozoite proteins than western blots probed with AS. Thus, the ability of AS to react with almost all of the corresponding immunogenic bands that were detected by IS in western blots, especially surface proteins 2, 3, 4 and P20, was considered a confirmation of the natural immunogenicity of these polypeptides.

In light of the apparent strong immunogenicity and surface localization of P20, this data indicates that P20 may be immunologically important. Augustine and Danforth (4) described a MAb that cross-reacted with a  $M_r$  24,000 sporozoite surface antigen from 4 different poultry-specific Eimeria species. This indicates that certain surface antigens may be widely distributed among the Eimeria (4).

Protective antigens of several species of Plasmodium have been found to be associated with the sporozoite surface membrane (13, 73, 79, 129). Whether any of the immunogenic E. bovis sporozoite antigens are protective remains to be determined.

In the present study, immunoelectron microscopy (IEM) was used to show that parasite-specific antibodies in AS and IS reacted 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 prefixed E. bovis sporozoites. This indicates that all of these parasite structures are antigenic. Since sporocysts and sporozoites develop within oocysts, they likely share common or similar antigens. This would help explain why oocysts, sporocyst residua and walls react with IS when these structures would not normally be expected to stimulate an immune response due to their sedentary existence in the gut of host animals. On the other hand, these structures may provide sufficient antigenic stimulus for the induction of gut immune responses and the subsequent production of immunoglobulins (7, 125). Work in our laboratory has shown that a crude E. bovis oocyst antigen is capable of inducing in vitro antigenic-specific mitogenesis of bovine peripheral blood mononuclear cells (Dr. H.P.A. Hughes, unpublished data).

Detection of parasite-specific antibody in AS or IS by IEM correlated well with results obtained by IFA assay except that IEM detected specific IgG bound to the inner surface of the sporocyst wall and residuum of E. bovis, whereas in IFA assays, these parasite structures exhibited little or no fluorescence. IEM showed that receptors for polyclonal IgG were most numerous on the inner surface of sporocyst walls, but few or no receptors were present on the outer surface. Whitmire and Speer (126) also found that IgG in immune serum localized on the inner surface of sporocysts of E. falciformis. Sporocysts of E. bovis and E. falciformis are similar ultrastructurally with both being composed primarily of a single unit membrane. It is interesting that both of these species of Eimeria express immunogenic antigens on the inner but not outer surface of their sporocyst walls. In contrast, Speer et al. (116) found that monoclonal IgG against E. tenella was localized primarily on the outer surface of its sporocysts. Ultrastructurally, sporocysts of E. tenella are composed of an inner layer of moderately electron-dense material and a unit membrane makes up the outer layer.

Sporozoites of E. bovis that were prefixed in glutaraldehyde reacted less with EbS9 or EbS11 than did those exposed to EbS9 or EbS11 prior to glutaraldehyde fixation. Thus, prefixation in glutaraldehyde evidently

alters the epitopes so that most of the P20 antigen in the sporozoite plasmalemma fails to react with EbS9 and EbS11.

As expected, less localization of ferritin occurred on EbS9 or EbS11-treated sporozoites than on those exposed to polyclonal AS or IS. IEM confirmed the findings of live IFA and autoradiography that P20 is an integral or extrinsic protein of the sporozoite plasmalemma. EbS9 and EbS11 did not react with oocyst walls by IEM indicating that these parasite structures lack the P20 antigen.

Live E. bovis sporozoites that were exposed to AS or IS and then fixed, capped and subsequently shed immune complexes at their posterior ends. This capping process was similar to that described for mammalian cells as well as various protozoan parasites such as Trypanosoma spp., Leishmania spp. and Eimeria spp. (5, 9, 11, 12, 22, 24, 117, 126). However, tachyzoites of Toxoplasma gondii appear to cap and then shed immune complexes from their anterior ends (25). Yet, during penetration of host cells, Dubremetz et al. (23) observed that tachyzoites of T. gondii capped and shed immune complexes from their posterior ends at the level of the moving junction between host cells and penetrating tachyzoites.

In contrast to AS and IS, live sporozoites that were exposed to EbS9 or EbS11, for up to 45 min prior to fixation, did not cap or shed P20-MAb immune complexes. Fixed antigenic sites of T. gondii and E. nieschulzi

demonstrate that not all immune complexes on parasite surfaces are capable of being capped or subsequently shed (11, 23). It is possible that P20 represents one such antigenic molecule since EbS9 or EbS11 did not induce capping. However, Speer et al. (117) described posterior capping and shedding of immune complexes that were partially comprised of MAbs by E. tenella sporozoites. In this case, cap formation was not induced by MAb alone but only in the presence of a second antibody ligand in the form of ferritin- or colloidal gold-conjugated antibody (117). The necessity of a double antibody reaction to induce capping may indicate that the individual antigens to which the MAbs are directed are spaced far enough apart in the sporozoite plasmalemma to prevent crosslinkage. Thus, the necessary crosslinking lattice could not be formed by MAb alone whereas the second labelled antibody would allow for sufficient crosslinking (117). This is likely to have occurred with EbS9 and EbS11 on E. bovis sporozoites. Lack of crosslinking by MAbs may also have resulted from no more than 1 available epitope on a specific antigen for MAb binding (5). Moreover, the dimensions of the exposed antigenic molecule may not have permitted more than 1 MAb molecule to bind or the spatial arrangement of specific antigens with other molecules may conceal many antigenic determinants from monovalent MAbs (5). Conversely, the multivalency of the antibodies present in AS or IS would be

capable of inducing sufficient antigen-antibody lattice formation to cause capping.

Sporozoites of E. bovis capped immune complexes relatively rapidly (within 5 min) after exposure to AS and IS. After 10 or 20 min, most sporozoites had shed immune complexes. These time intervals are similar to those described for polyclonal IgG-induced capping in Trypanosoma spp. and Leishmania enrietti (12, 22). Capping by E. bovis sporozoites also appears to be similar to the circumsporozoite precipitation (CSP) reaction described for several species of Plasmodium after exposure to immune serum (13, 73, 123). However, the CSP reaction results in the lysis of Plasmodium sporozoites after 30 min, whereas no lysis of live E. bovis sporozoites was observed after they had been exposed to heat-inactivated AS, IS or MAbs for up to 45 min (123). Except for the loss of some plasmalemma and cytoplasm that formed vesicles at the parasites' posterior, no ultrastructural alterations were detected in sporozoites treated with AS or IS. The extruded cytoplasm did not contain organelles and appeared to be surrounded by parasite plasmalemma. This finding is similar to that of Wright et al. (128) who reported on the release of long finger-like streamers of cytoplasm (called plasmanemes) enclosed with Trypanosoma brucei plasmalemma but without intracellular organelles. Several authors have suggested that plasmanemes are probably artifacts that are caused by in vitro

manipulation of parasites (26). Since only a few E. bovis sporozoites exposed to AS or IS were observed with extruded cytoplasm, such an event must evidently occur only in some parasites, occur only momentarily or be due to artifact.

If sporozoites of E. bovis had been exposed to AS or IS for longer periods than those tested (i.e., 45 min) then perhaps the sporozoites would have completely capped and shed all immune complexes from their surfaces. Sporozoites of E. falciformis and E. tenella completely capped and shed immune complexes within 15 and 30 min, respectively, after exposure to IS or MAbs (117, 126). Since sporozoites move in an anterior direction, motility may facilitate capping of immune complexes to the parasite posterior (117). Almost all motile mammalian cells studied to date have been shown to cap cross-linked surface antigens while most nonmotile cells do not (9).

Additional studies are needed to elucidate whether E. bovis sporozoites re-insert identical or different surface antigens into their plasmalemma following capping and shedding of immune complexes. The insertion of novel antigens could conceivably allow these parasites to evade existing immune responses. However, previous studies have shown that the surface antigens of Trypanosoma spp. and Leishmania enrietta which have been capped and shed are replaced with identical antigens (5, 22).

EbS9 and EbS11 were the only 2 MAbs generated against E. bovis that caused a decrease in penetration of MDBK cells by sporozoites. When compared to controls, the percent decrease in sporozoite penetration was 79 and 73% for EbS9 and EbS11 pretreated sporozoites, respectively. EbS9 and EbS11 may have caused a similar percent decrease in host cell penetration due to similar reactivities by these two MAbs toward the P20 antigen. Augustine and Danforth (3) found that treating sporozoites of E. tenella and E. adenoides with surface-reacting MAbs decreased penetration of cultured cells by 37 to 63%. As expected, in the present study, the nonsurface-reacting MAbs did not cause an appreciable decrease in sporozoite penetration. Furthermore, pretreatment of MDBK cells with EbS9 or EbS11 had no adverse effect on sporozoite penetration. MAbs have been found to inhibit in vitro adherence of Entamoeba histolytica trophozoites to mammalian cells by as much as 86% (80). However, no host-cell receptors or adherence mechanisms have been proven to exist for Eimeria species.

Pretreatment of E. bovis sporozoites with IS did not cause a significant decrease in the number of sporozoites penetrating MDBK cells, and with the exception of the 1/2 dilution of AS, similar results were obtained from AS pretreated sporozoites. However, if these effects were dependent on the concentration of parasite-specific antibody alone, they should have been titratable. Thus, it appears

that the significant decrease in sporozoite penetration that was caused by pretreatment with a 1/2 dilution of AS was probably due to experimental error, since undiluted AS had no significant effect. The slight decrease in host cell penetration that was displayed by the AS pretreated sporozoites compared to IS pretreated sporozoites may have resulted from the increased AS reactivity towards sporozoite surface antigens as detected by immunoblots. However, the differences in group variances do not allow for a more exact comparison between AS and IS pretreated sporozoites.

Several attempts were made to determine the effects of AS, IS and MAbs on the ability of sporozoites of E. bovis to undergo merogony in MDBK cells. Since it was difficult to maintain MDBK cell cultures for as long as 2 weeks (the length of time needed to assess meront development), M617 cells were used instead because they support large numbers of meronts and the cultures can be maintained easily for relatively long periods (113).

Because M617 cells were originally isolated from a bovine monocytic cell line, the number of intracellular sporozoites that remained 10 days ASI of these cells were tabulated to determine whether phagocytosis, opsonization or intracellular killing of antibody-treated sporozoites had occurred. Evidently, opsonization of sporozoites by EbS9 and EbS11 did not occur since there were no significant differences in numbers of intracellular sporozoites between

experimental and control groups. If phagocytosis and/or intracellular killing of sporozoites had occurred, then it was independent of MAb treatment. Pretreatment of E. bovis sporozoites with EbS9 or EbS11 resulted in an 89 and 94% decrease in meront development 10 days ASI of M617 cells, respectively. These numbers represent only a 10 to 20% greater decrease than that afforded by these MAbs to inhibit sporozoite penetration of MDBK cells. Since Reduker and Speer (84) determined that approximately 80% of intracellular E. bovis sporozoites will proceed to develop in M617 cells under optimal conditions, it is likely that the decreases in meront development in the present study were not due to the direct effects of EbS9 or EbS11. Thus, EbS9 and EbS11 interfered with the ability of E. bovis sporozoites to penetrate M617 cells but had no deleterious effect on the ability of those that did penetrate to develop into meronts.

At least in the case of EbS11 pretreated sporozoites, the decrease in penetration (and hence development) was titratable. Pretreatment of sporozoites with EbS11 that had an IFA titer of 10 (instead of 20 as used in previous experiments), resulted in little or no significant decrease in numbers of meronts. These results imply that the decrease in meront development was dependent on the concentration of EbS11 (and presumably, EbS9) since pretreatment of sporozoites with EbS9 or EbS11 with IFA titers of 20

resulted in a highly significant decrease in meront development.

Pretreatment of E. bovis sporozoites with AS did not decrease sporozoite development in M617 cells as compared to NS controls. This result correlates well with the previous finding concerning the inability of AS to appreciably decrease penetration of MDBK cells. However, IS pretreatment of sporozoites completely abolished their ability to undergo further development in M617 cell cultures. This result was surprising since IS did not inhibit sporozoite penetration of MDBK cells. Even though AS reacted to most of the immunogenic sporozoite proteins that were also detected by IS, it did not cause a significant decrease in meront development. Thus, IS must contain a factor that has an inhibitory effect on meront development which is not present in AS.

Speer et al., (113) and Hughes et al., (43) reported that a lymphokine(s) from concanavalin A-stimulated bovine peripheral blood T-cells was capable of inducing M617 cells to inhibit merogony and to kill intracellular forms of E. bovis, E. papillata and T. gondii. Perhaps the IS obtained in the present study from a normal E. bovis infection contained a similar factor or lymphokine that induced the M617 cells to inhibit and kill intracellular stages of E. bovis. Evidently, AS that was raised by IV inoculation of sporozoites did not contain the factor. Similar studies

need to be performed using IS and AS with a nonphagocytic bovine epithelioid cell such as MDBK.

In vivo studies need to be performed with EbS9, EbS11, AS and IS treatment of E. bovis sporozoites especially since Rose (92) found that E. maxima sporozoites incubated with heat-inactivated immune chicken serum caused a 46% decrease in oocyst-output when inoculated into nonimmune birds.

The differences between EbS9 or EbS11 and AS or IS reactivity are notable since EbS9 and EbS11 pretreatment of E. bovis sporozoites significantly inhibited penetration of cultured cells by sporozoites, whereas AS and IS did not. Additionally, sporozoites were shown to cap and shed AS- or IS-derived immune complexes but not those comprised of EbS9 or EbS11. Based on these results, the inability of sporozoites to cap immune complexes containing EbS9 or EbS11 may have interfered with their ability to penetrate cells.

Motility in mammalian cells appears to be dependent on a polarized endocytic cycle of the cell and it is generally accepted that motility must be present in order for cells to undergo capping (9). Cells insert newly synthesized membrane at their leading edges and reabsorb plasmalemma at their trailing edges, which causes a net movement of plasmalemma towards the cell posterior along with any possible crosslinked antigens (9). Thus, sporozoites treated with EbS9 or EbS11 may have been unable to retain their motility because of their inability to cap, shed and/or reabsorb

components of the plasmalemma at their posterior ends. This is likely to have resulted in few intracellular sporozoites since motility is required for sporozoite penetration of cells (110). On the other hand, sporozoites of E. bovis which were able to cap and shed immune complexes derived from exposure to AS or IS may have retained their motility and, therefore, ability to penetrate cells. Stewart et al. (118) demonstrated that sporozoites of P. berghei which were rendered immobile by a MAb directed against the repeating immunodominant epitope of the circumsporozoite protein lost their ability to penetrate cells (118).

Since certain parasite antigens appear to be needed for motility and host cell penetration by E. bovis sporozoites, further studies are necessary to comprehend this event. A better understanding of host cell penetration will undoubtedly accelerate our ability to prevent this crucial step in the life cycle of coccidian parasites.

## SUMMARY

Eimeria bovis has been reported to be the most frequent cause of bovine coccidiosis in the U.S.A. as well as other parts of the world. Clinical signs of coccidiosis include severe hemorrhagic diarrhea which may ultimately lead to the death of infected animals. Surviving animals are usually resistant to reinfection, however, to date, there are no vaccines or satisfactory prophylactic measures for bovine coccidiosis. Identification of immunogenic parasite antigens as well as the production of parasite-specific MAbs might eventually lead to isolating and elucidating the roles of various antigens in immune responses to bovine coccidiosis.

Holstein-Friesian bull calves that survived an initial oral inoculum of  $3.5 \times 10^4$  sporulated E. bovis oocysts displayed no overt symptoms of coccidiosis upon oral challenge with  $5 \times 10^4$  sporulated oocysts (a lethal dose in nonimmune calves). These animals demonstrated a primary and secondary humoral response to initial and challenge inoculations, respectively, and passed only a single oocyst per gram of wet feces during the interval after the challenge inoculum that corresponded to the period of peak oocyst discharge for initial infections.

Twelve MAbs that were elicited by whole E. bovis sporozoite antigen demonstrated at least 4 different IFA fluorescence patterns on acetone-fixed sporozoites. Three of these MAbs also cross-reacted by IFA with acetone-fixed first-generation merozoites and 2 MAbs, EbS9 and EbS11, reacted with the surfaces of live sporozoites.

Western blots of nonreduced E. bovis sporozoites which were probed with concentrated EbS9 or EbS11 revealed that both EbS9 and EbS11 reacted with the same or similar  $M_r$  20,000 protein band (P20). The antigenic determinants of P20 against which these 2 MAbs were directed appeared to be dependent on the tertiary structure of the polypeptide, since neither MAb reacted in western blots with reduced E. bovis sporozoites.

Radioiodination of nonreduced E. bovis sporozoites with  $^{125}\text{I}$  using lactoperoxidase followed by autoradiography confirmed the localization of P20 on the sporozoite surface. Autoradiography also revealed the presence of 5 additional sporozoite surface proteins. Western blots of nonreduced sporozoites that were probed with AS reacted intensely with P20 as well as 4 (proteins 2-5) of the 5 additional sporozoite surface proteins, whereas IS also reacted intensely with P20 but only weakly with 3 (proteins 2, 3 and 4) of the other 5 surface proteins. These results demonstrate the inherent immunogenicity of P20 and indicate that

this sporozoite surface molecule may be immunologically important.

IEM revealed that IgG in AS and IS reacted 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 prefixed E. bovis sporozoites. This indicates that all of these parasite structures possess immunogenic antigens. Postfixed sporozoites were observed to cap and subsequently shed AS and IS immune complexes at their posterior ends. Even though EbS9 and EbS11 were observed to react with sporozoite plasmalemmae by IEM, sporozoites did not shed immune complexes derived from these MAbs.

Sporozoites of E. bovis were shown to invaginate the host cell plasmalemma during penetration and to become surrounded momentarily by it. This membranous envelope derived from the host cell plasmalemma was apparently short-lived since sporozoites were found with only short segments of host cell membrane around them or free in the host cell cytoplasm at 5 or 10 min ASI. Thus, the parasitophorous vacuole membrane that later surrounds the sporozoites and meronts is probably derived from the host cell endoplasmic reticulum and not the plasmalemma.

In cultures of M617 cells, EbS9 reacted with a granular material within the parasitophorous vacuole surrounding first-generation meronts of E. bovis. This material may

represent P20 that is shed by developing meronts. Yet, there was no evidence of this material in the parasitophorous vacuole surrounding meronts in EbS9-treated MDBK cells. EbS9 did not react with the parasitophorous vacuole membranes or first-generation merozoites in either cell line.

Pretreatment of E. bovis sporozoites with EbS9 or EbS11 resulted in a 79 and 73% decrease in sporozoite penetration of MDBK cells, respectively. Pretreatment of sporozoites with nonsurface-reacting MAbs resulted in no decrease in sporozoite penetration of cultured MDBK cells. Likewise, AS or IS pretreatment of sporozoites had no effect on sporozoite penetration of MDBK cells.

EbS9 or EbS11 did not effect the ability of sporozoites of E. bovis to develop to meronts in M617 cells. Other nonsurface-reacting MAbs or AS caused no decrease in intracellular development of meronts of E. bovis. However, pretreatment of sporozoites with IS completely inhibited the ability of E. bovis sporozoites to develop to first-generation meronts. Inhibition of meront development by IS may have been due to factors other than parasite-specific antibodies within the IS. No ultrastructural alterations were detected in sporozoites that were treated with AS, IS or MAbs.

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