MONTANA STATE UNIVERSITY

Immunoelectron microscopy of two Eimeria bovis antigens

by Kehming Liaw

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Veterinary Science
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Abstract:
Immunofluorescence and immunoelectron microscopy were used to study the localization of antigens of sporozoites and first-generation merozoites of E. bovis. Monoclonal antibody EbS9, which reacts against a 20,000 molecular weight (called P20), reacted primarily with the external surface of the parasite plasmalemma and with the necks of sporozoite rhoptries which are components of the apical complex. As sporozoite underwent merogony within cultured bovine monocytes (M617 cells), P20 was shed gradually into the parasitophorous vacuole and was absent in first-generation merozoites. EbS15 also reacted against a phosphorylase 97.4 kD molecular weight marker indicating that parasite polypeptide shares common epitopes with phosphorylase. In immunoelectron microscopy studies, EbS15 appeared to react with amylopectin granules, especially the moderately electron-dense material associated the cytoplasm surface of the granules. At I hr after sporozoite inoculation (ASI) of M617 cells, EbS15 reacted intensely with amylopectin granules, whereas at one day ASI most sporozoites had depleted their amylopectin. EbS15 reacted with low intensity with amylopectin in meronts and merozoites, indicating that a change had occurred in the epitope recognized by EbS15 in the amylopectin granules or the phosphorylase enzyme associated with amylopectin granules.

This study also involved the development of ultrastructural techniques for the internal localization of parasite antigens. Antigenicity and preservation of ultrastructural feature was far superior in those specimens fixed in OsO4 and embedded in Spurr's medium; embedding in LR White produced unsatisfactory results.

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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

Immunofluorescence and immunoelectron microscopy were used to study the localization of antigens of sporozoites and first-generation merozoites of E. bovis. Monoclonal antibody EbS9, which reacts against a 20,000 molecular weight (called P20), reacted primarily with the external surface of the parasite plasmalemma and with the necks of sporozoite rhoptries which are components of the apical complex. As sporozoite underwent merogony within cultured bovine monocytes (M617 cells), P20 was shed gradually into the parasitophorous vacuole and was absent in first-generation merozoites. EbS15 also reacted against a phosphorylase 97.4 kD molecular weight marker indicating that parasite polypeptide shares common epitopes with phosphorylase. In immunoelectron microscopy studies, EbS15 appeared to react with amylopectin granules, especially the moderately electron-dense material associated the cytoplasm surface of the granules. At 1 hr after sporozoite inoculation (ASI) of M617 cells, EbS15 reacted intensely with amylopectin granules, whereas at one day ASI most sporozoites had depleted their amylopectin. EbS15 reacted with low intensity with amylopectin in meronts and merozoites, indicating that a change had occurred in the epitope recognized by EbS15 in the amylopectin granules or the phosphorylase enzyme associated with amylopectin granules.

This study also involved the development of ultrastructural techniques for the internal localization of parasite antigens. Antigenicity and preservation of ultrastructural feature was far superior in those specimens fixed in OsO_4 and embedded in Spurr's medium; embedding in LR White produced unsatisfactory results.

INTRODUCTION

General

Eimeria bovis is the most common cause of coccidiosis in cattle in the United States. It also occurs in the ox, zebu, and water buffalo (1). Levine and Ivens reported that most of the coccidia of cattle produce some pathogenic effect on their hosts (2). Thirteen species of *Eimeria* occur in cattle of which *E. zuernii* and *E. bovis* are considered to be the most pathogenic. Severe clinical cases usually exhibit hemorrhagic enteritis and diarrhea with the feces containing stringy masses of mucus and clotted blood. Acutely infected animals usually suffer from loss of appetite, dehydration, and general weakness (3) which may lead to morbidity and high mortality, especially in young animals. Approximately 5-20% of the cattle treated for bovine coccidiosis die from the infection (4,5). In 1972, Fitzgerald estimated that bovine coccidiosis caused an annual worldwide monetary loss of 472 million dollars (6).

Eimeria bovis belongs to the Subkingdom Protozoa, Phylum Apicomplexa, Class Sporozoasida, Subclass Coccidiasina, Order Eucoccidiorida, Suborder Eimeriorina, Family Eimeriidae, Genus *Eimeria*, Species *bovis* (7). The

life cycle of E. bovis includes three phases: schizogony (merogony), gametogony, and sporogony (8). Animals become infected by ingesting sporulated oocysts, each of which contains eight sporozoites. Upon exposure to CO_2, trypsin and bile, the sporozoites excyst from the oocyst and actively penetrate the intestinal mucosa and localize intracellularly in endothelial cells of the central lacteal of the ileal villi, especially in a region 1 to 2 feet anterior to the ileocecal valve. At this location, the sporozoite undergoes first-generation schizogony (merogony) to form merozoites. These first-generation merozoites travel to the cecum and large intestine where they develop within glandular enterocytes to second-generation merozoites, which in turn form gamonts (also called gametocytes). Male gamonts (called microgamonts) produce approximately 100 microgametes, whereas each female gamont (macrogamonts) becomes a large spheroid form which does not multiply. Microgametes are tiny, biflagellated and motile, and actively seek and fertilize the larger macrogametes. Soon after fertilization, the zygote develops into an oocyst by forming a chemically resistant wall at its surface. The oocyst causes destruction of its host enterocyte and is released into the intestinal lumen and voided in the feces. In the presence of moisture and oxygen the oocyst undergoes sporulation to form eight sporozoites. The parasite can

repeat its life cycle when ingested by other suitable hosts (8,9).

The ultrastructure of the sporozoites and merozoites of E. bovis is typical of the Apicomplexa (2) in that both possess an apical complex consisting of 2 apical rings, 2 polar rings, a conoid, 22 subpellicular microtubules, rhoptries, and micronemes. The ducts of the micronemes apparently run anteriorly into the rhoptries or join a common duct system with the rhoptries, which in turn lead to the zoite surface at the anterior tip (2,10). Rhoptries and micronemes appear to have similar contents in electron micrographs, and it is thought that this material is secreted by sporozoites and merozoites to facilitate their penetration into host cells (11-14).

Some researchers have suggested that lytic enzymes or other substances secreted by rhoptries and micronemes may play a important role in cell penetration (14-16). In Plasmodium berghei, multilamellar membranous whorls are found both associated with elements of the rhoptry-microneme complex and closely apposed to the external surface of the sporozoite (17,18), indicating that phospholipids containing materials may be secreted from the rhoptry-microneme complex which facilitate the penetration of the parasite into the host hepatocyte. Furthermore, it has been suggested that the rhoptry-microneme complex discharges materials which become

lamellar bodies that 1) attach to the external surface of sporozoites and merozoites and facilitate the invasion of these stages into host cells and 2) contribute to the formation of the parasitophorous vacuole (19,20). Scholtyseck and Mehlhorn (21) suggested that micronemes are involved in the production of osmiophilic rhoptry materials and that the rhoptry discharges these materials extracellularly at the anterior tip of the sporozoite and merozoite.

A penetration enhancing factor (PEF) has been extracted from tachyzoites of T. gondii or from the medium of cultured cells in which T. gondii tachyzoites were grown (22). The PEF appears to enhance entry of tachyzoites into cultured mammalian cells (22). Although some enzymes, such as lysozyme and hyaluronidase, have also been found to enhance tachyzoite penetration (23), PEF is relatively more active per unit weight and is thought to be a specific protein that is secreted by rhoptries and micronemes during the process of cell invasion (24). Monoclonal antibodies that react against rhoptries can also inhibit the effects of PEF, indicating that the rhoptry is the likely site of PEF storage (25).

In the case of Eimeria spp., numerous authors have hypothesized that rhoptries are secretory organelles that may facilitate the penetration process (21-26). Most of these hypotheses were based on ultrastructural studies.

In E. magna, sporozoites fixed in the process of penetrating cultured bovine trachea cells exhibited empty, or partially empty, rhoptries, which evidently had released their contents during penetration.

The rhoptries of Eimeria spp. as well as those of other coccidia may contain substances similar to the histidine-rich polypeptides believed to be associated with the rhoptries of Plasmodium lophurae (27). Such polymers of basic amino acids can produce numerous changes in cell membranes such as the loss of structural rigidity, increased permeability, and membrane disorganization (28,29). Shotton et al (30) showed that polycationic polypeptides produced an aggregation of the protein constituents of erythrocyte membranes that resulted in the blebbing of numerous vesicles from the lipid bilayer. Another possible explanation was provided by Bannister et al,(31) in a study on penetration involving Plasmodium knowlesi merozoites into monkey erythrocytes. They suggested that secretory products from the rhoptries or micronemes may have been incorporated into the host cell membrane which caused disorder in the phospholipid bilayers, resulting in the inward expansion of the host cell membrane and penetration of the parasite.

The rhoptries from T. gondii tachyzoites are known to be derived from the Golgi complex (32), which is formed in part from the nuclear envelope (33). Although the

origin of rhoptries in malarial and eimerian parasites is still not known, they may have origins similar to that of T. gondii.

In general, parasites utilize three mechanisms to evade the full effects of the host immune responses. 1. Location: Some parasites such as Trypanosoma cruzi and Plasmodium spp. escape detection by the host immune system via their anatomical inaccessibility (34). Trypanosoma cruzi can escape immune surveillance within macrophages by destroying the parasitophorous vacuole to become situated free in the cytoplasm, thus avoiding being destroyed by lytic enzymes within phagolysosomes. Plasmodium spp. are protected during erythrocytic schizogony by being enclosed within a membrane-bound bag of hemoglobin, the red blood cell. Other parasites have developed mechanisms of living within macrophages by avoiding destruction by O_2 metabolites and lysosomal enzymes. T. gondii appears to avoid triggering the oxidative burst while other protozoan parasites destroy products of the oxidative burst (35). 2. Avoidance of recognition: Parasites may use various mechanisms to avoid recognition by the host even if they are exposed to parasite-specific antibody. For example, the African trypanosomes undergo antigenic variation in which they change the glycoproteins of their surface coat enabling them to escape immune surveillance. Other parasites, such as Schistosoma spp. acquire a surface

layer of host antigens so that the host cannot distinguish them from self. The host antigens acquired by Schistosoma spp., include surface molecules containing A, B and H specific blood determinants or antigens of the major histocompatibility complex. 3. Suppression of the host immune response: Parasites may produce biologically active molecules which have immunosuppressive effects on the host. These parasite molecules may cause their immunosuppressive effects by 1) combining with antibodies, and, thus diverting them away from the parasites; 2) blockading effector cells either directly or by forming immune complexes; 3) by inducing B or T cell tolerance, presumably by blockade of antibody-forming cells or by depletion of mature antigen-specific lymphocytes (i.e. clonal exhaustion); 4) polyclonal activation of numerous B lymphocyte populations leading to impaired B cell function; 5) activating suppressor cells, which may be T cells or macrophages or both (36-39).

There are several other mechanisms that parasites may use in order to escape deleterious effects of the immune system. Roberts et al. (40) found that sporozoites of Eimeria larimerensis entered and exited several cells before finally remaining intracellular and undergoing further development. When sporozoites exited cells, they carried with them a thin layer of host cell cytoplasm as well as the host cell plasmalemma. This is also the case

with E. bovis in which the sporozoite carries a host cell envelope from one cell to the next (Speer, unpublished data). Sporozoites passing from one cell to another would be protected against the effects of antibodies by the envelope of host cell material.

Species of Eimeria, Trypanosoma, Leishmania, and T. gondii are also capable of escaping immune surveillance by capping and shedding antigen-antibody complexes from their surfaces (41,42).

Both humoral and cell-mediated immune mechanisms appear to be involved in resistance to reinfection by Eimeria spp., but the stages against which the host immune response is directed has not yet been determined. Recently, the research group studying bovine coccidiosis at the Veterinary Research Laboratory at Montana State University has published several reports concerning the effects of monoclonal antibodies (MAb) on sporozoites of E. bovis, the immunodominant surface antigens of E. bovis sporozoites and the shedding of a 20,000 molecular weight surface antigen by developing meronts of E. bovis (42-44). Similar reports have been published on T. gondii (25) and Plasmodium spp. (45-51). In studies with several Plasmodium spp., MAbs and polyclonal antibodies against the circumsporozoite proteins (CSP) have been found to inhibit sporozoite penetration of host cells. Even though these findings involve different genera and species,

further research may show that the antigens against which the neutralizing Abs react might be similar in amino acid sequence and composition.

Protective immunity to Plasmodium spp. appears to be mediated in part by antibodies directed against surface CSP. A similar situation may exist with antibodies directed against the surface of sporozoites of Eimeria spp. (7,42). Surface-reacting MAbs have been found to inhibit penetration of cultured cells by sporozoites of two avian coccidia, E. tenella and E. adenoides, and one bovine coccidium, E. bovis (7,42). Treatment of E. bovis sporozoites with either of two MAbs (EbS9 and EbS11) resulted in an approximately 75% decrease in sporozoite penetration of cultured cells (42). Both EbS9 and EbS11 reacted in western blots of solubilized E. bovis sporozoite with a 20,000 relative molecular weight (Mr) protein (called P20) which was also found to be an immunodominant surface antigen (43). MAbs EbS9 and EbS11 reacted against the anterior one third of acetone-fixed sporozoites which had lost the integrity of their plasmalemma allowing access by the MAbs to the sporozoite interior (42). Thus, precursors of the P20 molecule evidently occur internally in the apical region E. bovis sporozoites.

In Plasmodium knowlesi, precursors of protective surface antigens have been found in association with

micronemes and rhoptries in the apical regions of sporozoites (46). Although micronemes and rhoptries can be distinguished ultrastructurally, they are considered to be interconnected by a complex ductule system and to function as secretory organelles, the secretion of which is believed to facilitate parasite penetration of host cells (53). Thus, it is possible that the micronemes and rhoptries of E. bovis sporozoites serve to store and transport P20 to the anterior tip of the sporozoite, where it is secreted or inserted into the plasmalemma (42).

EbS9 and EbS11 also reacted with the apical end of E. bovis sporozoites indicating that components of the apical complex (i.e. micronemes and rhoptries) may contain P20 or precursors of P20. P20 appears to be a likely candidate as a component of a bovine coccidiosis vaccine; however since P20 is shed during meront develop (42), it is likely that an effective coccidiosis vaccine will require additional components. Another monoclonal antibody (EbS15) reacted against an internal antigen (PX), but not surface antigens of sporozoites and against a surface antigen (not an internal antigen) of first-generation merozoites of E. bovis (7). Thus, EbS9 and EbS15 reacted against different parasite antigens each of which is expressed differently by sporozoites and first-generation merozoites of E. bovis.

To date, no one has developed ultrastructural techniques suitable for the intracellular localization of antigens of sporozoites, meronts and merozoites of Eimeria spp. by monoclonal or polyclonal antibodies. In developing such techniques, several factors must be considered, such as preservation of the normal ultrastructure of the parasite as well as the antigenic epitopes against which the antibodies react.

Objectives

The objectives of this proposal are to: 1) develop ultrastructural techniques for the intracellular localization of antigens of Eimeria bovis sporozoites, meronts and merozoite by monoclonal antibodies and 2) determine the intracellular localization and fate of two antigens, P20 and an unknown antigen (PX), against which EbS9 and EbS15 react, respectively.

MATERIALS AND METHODS

Continuous Cell Cultures

An established cell line of bovine monocytes (M617) was used as host cells for the cultivation of sporozoites, meronts and merozoites of E. bovis. The M617 cell line was originally obtained from blood monocytes of a 6-year-old Guernsey cow. They are esterase positive and phagocytic with a normal karyotype, and do not express class II antigens of the major histocompatibility complex (G. A. Splitter, unpublished data). M617 cells were maintained in culture medium (CM) consisting of RPMI 1640 (GIBCO, Long Island, NY), 15% fetal bovine serum (FBS, Hyclone Laboratories, Inc., Logan, UT), and 2 mM L-glutamine, 50 U of penicillin G per ml and $5x10^{-2}$ mM 2-mercaptoethanol per ml.

Parasite

At 18 to 21 days after calves were orally inoculated with sporulated oocysts of E. bovis, their feces containing unsporulated oocysts of E. bovis were collected and passed through metal sieves to remove large fecal material. Oocysts of E. bovis were separated from the fecal debris by sugar flotation, centrifuged, and

sporulated in aerated aqueous 2.5% (w/v) $K_2Cr_2O_7$. Sporulated oocysts were then pooled and stored at 4°C in aqueous 2.5% $K_2Cr_2O_7$.

Sporulated oocysts were treated with 5.25% aqueous sodium hypochlorite for 1 hr at room temperature (RT) and centrifuged at 200xg for 10 min. The supernatant containing oocysts was diluted in Hanks' balanced salt solution (HBSS, pH 7.4; GIBCO, Santa Clara, CA), centrifuged to form a pellet of oocysts which was then washed several times with sterile HBSS to ensure removal of the sodium hypochlorite.

Sporulated oocysts which had been treated previously with sodium hypochlorite were suspended 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 a few 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, Detriot, MI; in HBSS, pH 7.4) for 3 hr in a 38°C water bath. Excysted sporozoites were washed once with HBSS, resuspended in HBSS, and passed through a nylon wool (Leuco-Pak, Fenwal Laboratories, Deerfield, IL) column in order to remove sporocysts, oocyst walls and oocysts (52).

The column eluate contained highly purified viable sporozoites and a few sporocysts, oocyst walls and oocysts.

Monoclonal Antibodies

MAbs EbS9 and EbS15 were obtained from stock solutions stored in the VRL Electron Microscope Facility. These MAbs were originally produced as described previously (42). Cultured medium from the cloned hybrids as well as heat-inactivated ascites fluid from pristine (Sigma)-primed BALB/cByJ mice inoculated with these hybridomas served as sources of ascites fluid containing parasite-specific MAbs. MAbs were concentrated from CM by precipitation in saturated ammonium sulfate (pH 7.2), dialyzed against distilled H_2O, and dissolved in 0.15 M phosphate-buffered saline (pH 7.4). Immunoglobulin classes and subclasses of the parasite-specific MAbs were determined with a commercial enzyme-linked immunosorbent assay murine-MAb isotyping kit (Hyclone Laboratories, Inc., Logan, UT). CM from unfused murine myeloma cells (Ag8) was processed as above, stored at -70^0C, and used as a control (7).

Indirect Immunofluorescence Assay

The indirect fluorescence antibody technique (IFA) used here was similar to that described by Paulin et al (52). M617 cells were grown on glass coverslips in 24-

well culture plates, inoculated with 10^6 E. bovis sporozoites, fixed and processed for IFA. M617 cells on coverslips were removed from the culture plates, fixed in absolute methanol at -20°C for 10 min, washed in phosphate-buffered saline solution (PBS), placed in a plastic petri dish cell side up and stored at -20°C. MAbs EbS9 and EbS15 were diluted 1:20, whereas the secondary FITC conjugated antimouse IgG antibody was diluted to 40 ug/ml with PBS. Each antibody was applied for 45 min at RT with three PBS washes performed between the first and second antibodies.

After incubation in the fluorescein-conjugated antibody, the coverslips were rinsed in PBS, drained and mounted on glass slides, using Mowiol 4-88 (Hoechst, Frankfurt, F.R.G) as a permanent mountant. To prevent drying, the coverslips were sealed at their edges with clear fingernail polish. Control specimens were prepared as described above except that MAbs EbS9 and EbS15 were replaced by MAb Ag8. Experimental and control specimens were examined by fluorescence microscopy (53).

Immunoelectron Microscopy

Specimens prepared for immunoelectron microscopy involved both pre-embedding and post-embedding techniques: 1) Pre-embedding method: Sporozoites ($7x10^6$) or merozoites ($1.5x10^7$) were prefixed in 0.15% (v/v)

glutaraldehyde in Millonig's phosphate buffer (MPB)(pH 7.4) for 20 min at RT, washed twice with HBSS, centrifuged, and then reacted with a 1:20 dilution of EbS9 or EbS15 monoclonals in PBS (pH 7.2) for 30-45 min at RT. Parasites were washed twice with PBS and then incubated with goat anti-mouse colloidal gold antibody (15nm) which was previously diluted 1:20 with PBS (pH 7.2) for 30 min at RT. After the parasites were washed twice in PBS and centrifuged at 1000 x g for 5 min the pellets were fixed with 2.5% (v/v) glutaraldehyde in MPB, treated with 1% OsO_4, dehydrated in ethanol, and embedded in Spurr's medium. Thin sections were stained with uranyl acetate and lead citrate, and examined with a JEOL 100CX transmission electron microscope (TEM)(54,55).

2) Post-embedding method: The two post-embedding protocols used in this study were a) 1% OsO_4 fixed, Spurr embedded and b) 1% glutaraldehyde fixed, LR White embedded.

Osmium tetroxide fixed, Spurr embedded: Infected M617 cells were washed with Sorensen's PBS three times, scraped from the flasks with a rubber policeman, decanted into 15 ml centrifuge tubes and centrifuged at 250 xg for 10 min. In an attempt to preserve parasite ultrastructure as well as antigenicity, the cells were prefixed for 30 min at RT in 0.5% glutaraldehyde, 1% acrolein and 0.2% sucrose in 0.075 M PBS buffer, washed twice with distilled

water and fixed in 1% OsO_4 for 1 hr (56). After fixation, the pellet was washed 3 times with cold distilled water (pH 7.2-7.4), dehydrated through an ethanol series, and then infiltrated and embedded in Spurr's medium as follows: 1 Spurr's : 1 absolute ethanol then 2 Spurr's : 1 absolute ethanol for 1 hr, specimens were then put in pure Spurr's for 12 hr at 4 ^{o}C. The Spurr's was then polymerized at 70 ^{o}C for 14 hr. Ultrathin sections were cut on a Sorvall MT 5000 ultramicrotome and picked up on nickel grids. Specimens on grids were placed on a drop of blocking buffer (4% bovine serum albumin [Sigma, St. Louis, MO] in 0.1 M PBS) for 10 min, immersed in the first antibody (EbS9 or EbS15) for 1 1/2 hr at RT, emersed in blocking buffer for 2 hr, treated with goat anti-mouse IgG conjugated with 15 nm colloid gold for 1 hr, and then treated with blocking buffer overnight. Specimens on grids were rinsed in PBS without BSA for 30 min, rinsed 10 min in distilled water, stained with uranyl acetate (5 mins) followed by lead citrate (3 mins), and viewed with a JEOL 100 CX electron microscope (56,57).

Glutaraldehyde fixed, LR White embedded: Infected M617 cells were washed with Sorensen's PBS three times, scraped from the culture flasks, decanted into 15 ml centrifuge tubes and centrifuged at 250 xg for 10 min, and then fixed with 1% glutaraldehyde in 0.1 M sodium cacodylate buffer at RT (58).

After fixation, the pellet was washed with PBS 3 times in 3 hr, then rinsed in PBS overnight. During day 2, the infected cells were partially dehydrated in 50% ethanol for 15 min, 70% ethanol with two one hr changes and infiltrated and embedded as follows: 2 LR White : 1 70% ethanol for 1 hr, pure LR White for 1 hr, and then pure LR White overnight. During the next day, specimens were placed in a third change of LR White for 1 hr and then into gelatin capsules filled completely with LR White resin. In order to conduct polymerization under anaerobic conditions, the gelatin capsules were sealed and incubated at 48°C for 30 hr. Ultrathin sections on nickel grids, were immersed in EbS9 or EbS15 for 1 hr at RT, rinsed in millipore-filtered PBS, and placed in goat anti-mouse Ig conjugated with 15 nm colloid gold for 15 min. Specimens on grids were washed in millipore-filtered PBS, air-dried, stained with uranyl acetate (5 min) and lead citrate (3 min) and viewed with a JEOL 100 CX electron microscope (56,58).

Polyacrylamide Gel Electrophoresis

Purified sporozoites and merozoites 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, 6.25×10^{-2} M Tris-HCl (pH 6.8)) at 100°C for 15 min at a ratio of 6×10^{6}

sporozoites to 10 ul of solubilizing solution (59). The sample as well as prestained molecular weight standards (BRL; Bethesda Research Laboratory, Bethesda, MD) were subjected to polyacrylamide gel electrophoresis (SDS-PAGE) in 10% polyacrylamide slab gels using a discontinuous buffer system as described by Laemmli (60). Following electrophoresis (25 mA for approximately 6 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. Sporozoite and merozoite proteins were 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.

Western Blotting and Immunodetection of Sporozoite and Merozoite Antigens on Nitrocellulose

Sporozoite and merozoite proteins were electrophoretically transferred from an SDS-polyacrylamide slab gel containing 10% acrylamide to nitrocellulose paper in a Trans-Blot Cell (Bio-Rad Laboratories, Richmond, CA)(61). Following transfer, the nitrocellulose sheet was fixed (20:10:70, methanol:acetic acid:distilled water) for 15 min (62), washed twice in distilled water, and incubated in bovine lacto-transfer technique optimizer for 1 hr at RT to block nonspecific binding sites (63). The nitrocellulose sheet was then probed with concentrated EbS15, or 15D6 (MAb against VSV, unpublished data)(diluted

1:500 in bovine lacto-transfer technique optimizer) in a moist chamber at 4°C overnight, followed by exposure at a 1:200 dilution of horseradish peroxidase-conjugated goat antimouse IgG (United States Biochemical Corp.) in bovine lacto-transfer technique optimizer. Bound peroxidase activity was developed with peroxidase substrate solution. (69). The M_rs of the sporozoite and merozoite antigens were estimated by comparing their R_fs to R_fs of prestained molecular weight standards (BRL, Bethesda, MD) which had been transferred to the same nitrocellulose sheet from the 10% SDS-polyacrylamide gel.

RESULTS

Immunofluorescence Microscopy

The immunofluorescence assay of methanol-fixed specimens, showed that both EbS9 and EbS15 reacted with sporozoites and meronts of E. bovis, but only EbS15 reacted with first-generation merozoites (Figs. 1-7). Sporozoites treated with EbS9 fluoresced strongly, especially in the anterior one-third of the parasite (Fig. 1). EbS15-treated sporozoites exhibited moderate fluorescence with most intense fluorescence located at the margin and a band just anterior to the equator of the parasite (Fig. 2). At 10 and 15 days after sporozoite inoculation of M617 cells, intermediate and mature first-generation meronts exhibited moderate fluorescence when treated with EbS9 or EbS15 (Figs. 3-5). For specimens treated with EbS9 and EbS15, the parasitophorous vacuole surrounding meronts contained a highly fluorescence, glandular material, especially in those treated with EbS9. Nearly mature and mature meronts treated with EbS9 showed little or no fluorescence, and intracellular and extracellular first-generation merozoites were negative (Fig. 6). In contrast to EbS9, meronts treated with EbS15 were moderately fluorescent except at their margins which

were highly fluorescent (Fig. 4). Also, intracellular and extracellular first-generation merozoites treated with EbS15 exhibited a speckled fluorescence patterns (Fig. 7).

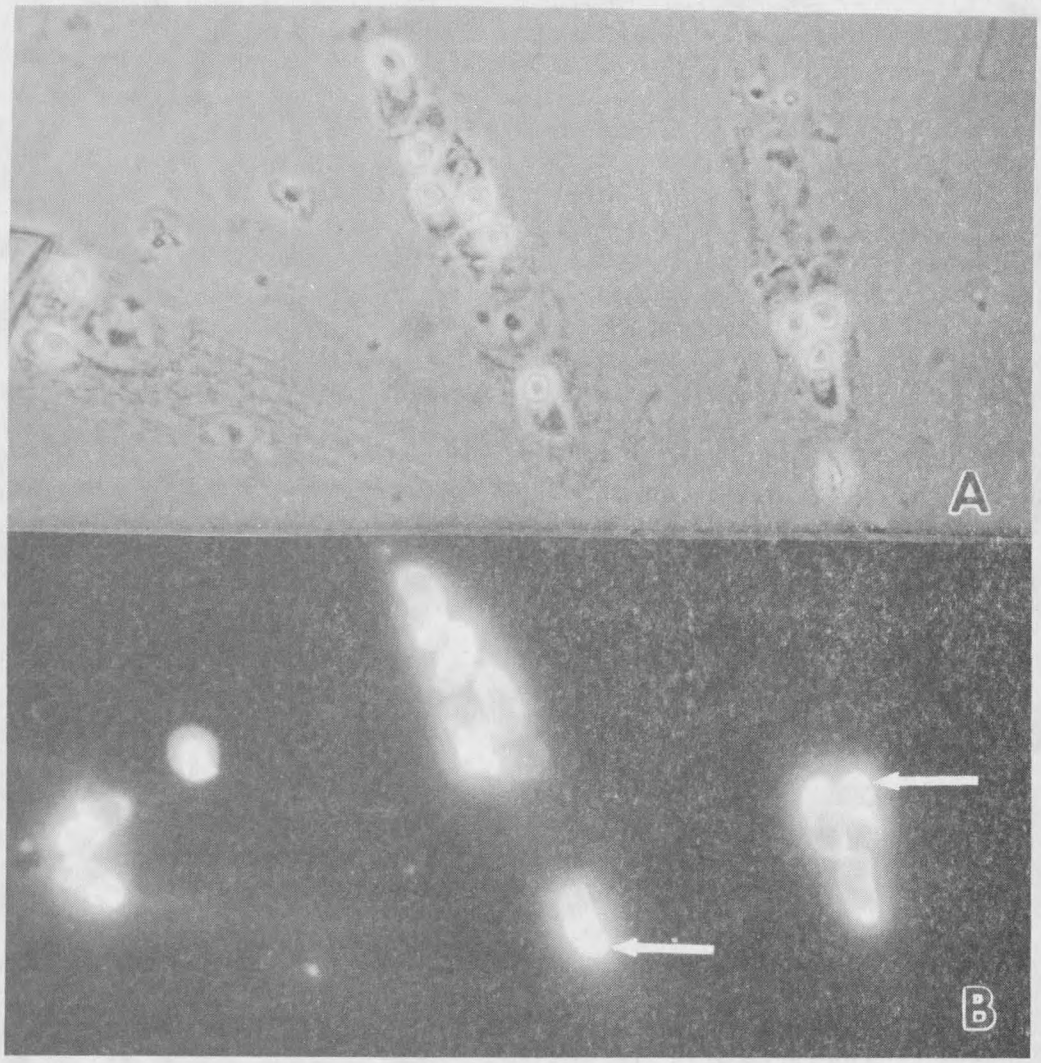

Fig. 1. A. Phase-contrast photomicrograph of several E. bovis sporozoites in M617 cells. X 630. B. Photomicrograph of IFA of the same specimens in A showing intense apical fluorescence of sporozoites of E. bovis (arrows). Treatment: methanol-fixed, EbS9, fluorescein-conjugated goat antimouse IgG. X 630.