



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
Montana State University
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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 OsO₄ and embedded in Spurr's medium; embedding in LR White produced unsatisfactory results.

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APPROVAL

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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 OsO₄ 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 CO₂, 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 an 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 O₂ 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 5×10^{-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, Detroit, 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 H₂O, 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°C, 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 (7×10^6) or merozoites (1.5×10^7) 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% OsO₄, 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% OsO₄ 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% OsO₄ 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 °C. The Spurr's was then polymerized at 70 °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_r s of the sporozoite and merozoite antigens were estimated by comparing their R_f s to R_f s 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).

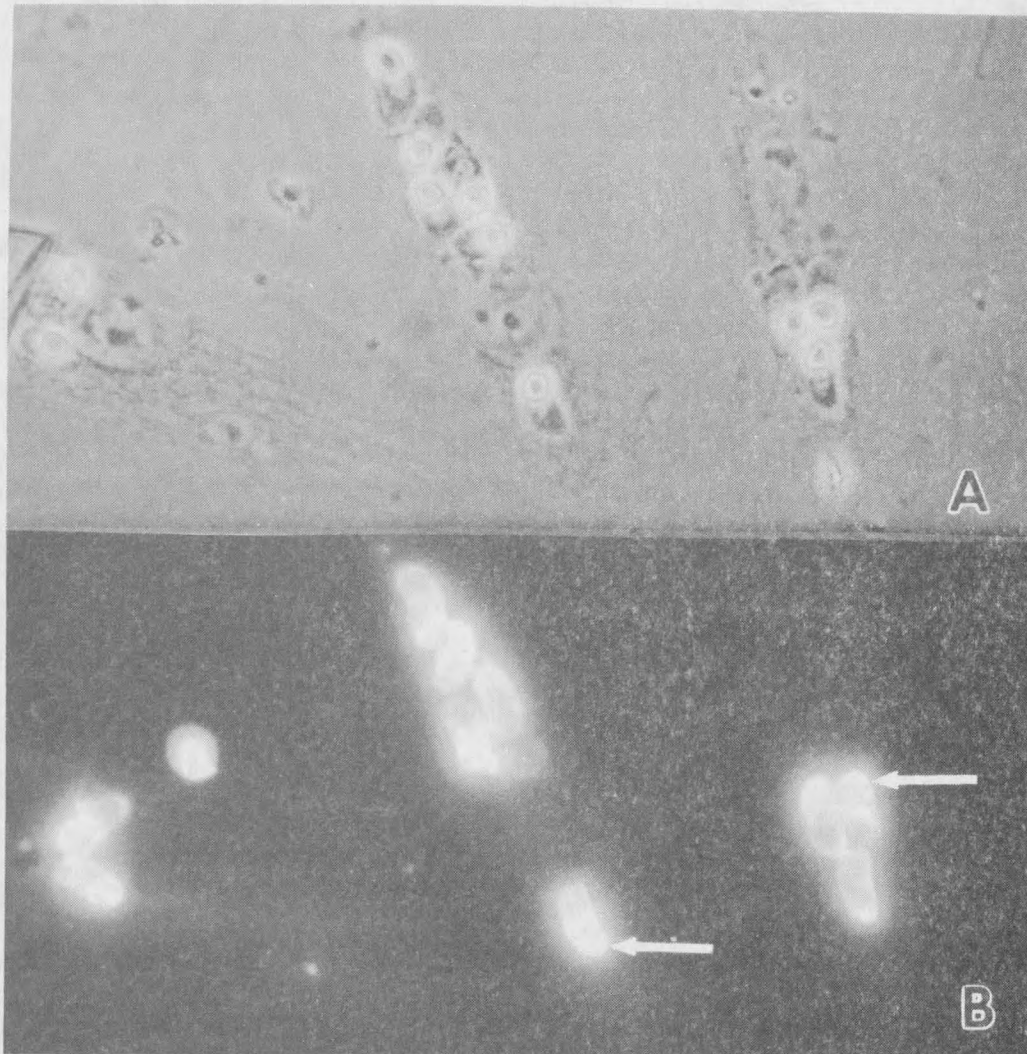


Fig. 1.

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.

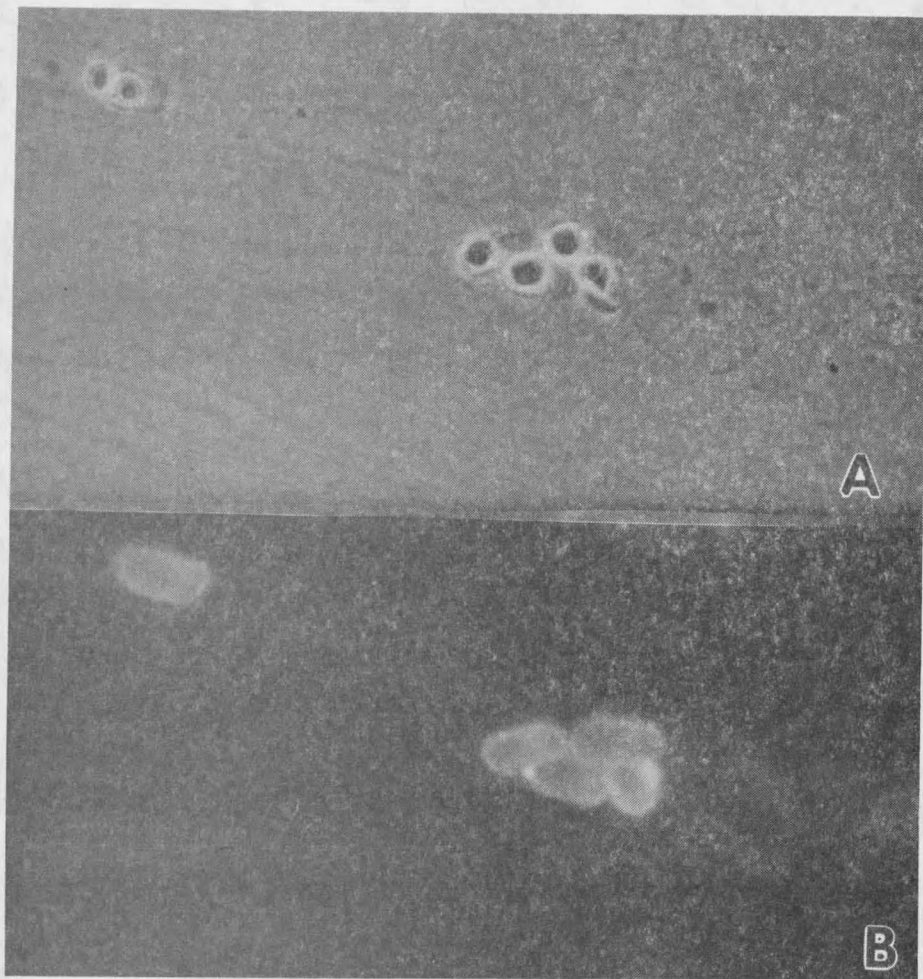


Fig. 2. A. Phase-contrast photomicrograph of five sporozoites in M617 cells. X 630.
B. Photomicrograph of IFA showing immunofluorescence patterns of EbS15 on sporozoites of the same specimens in A. Note the intense fluorescence mainly on both ends and central portion of sporozoite. Treatment: methanol-fixed, EbS15, fluorescein-conjugated goat antimouse IgG. X 630.

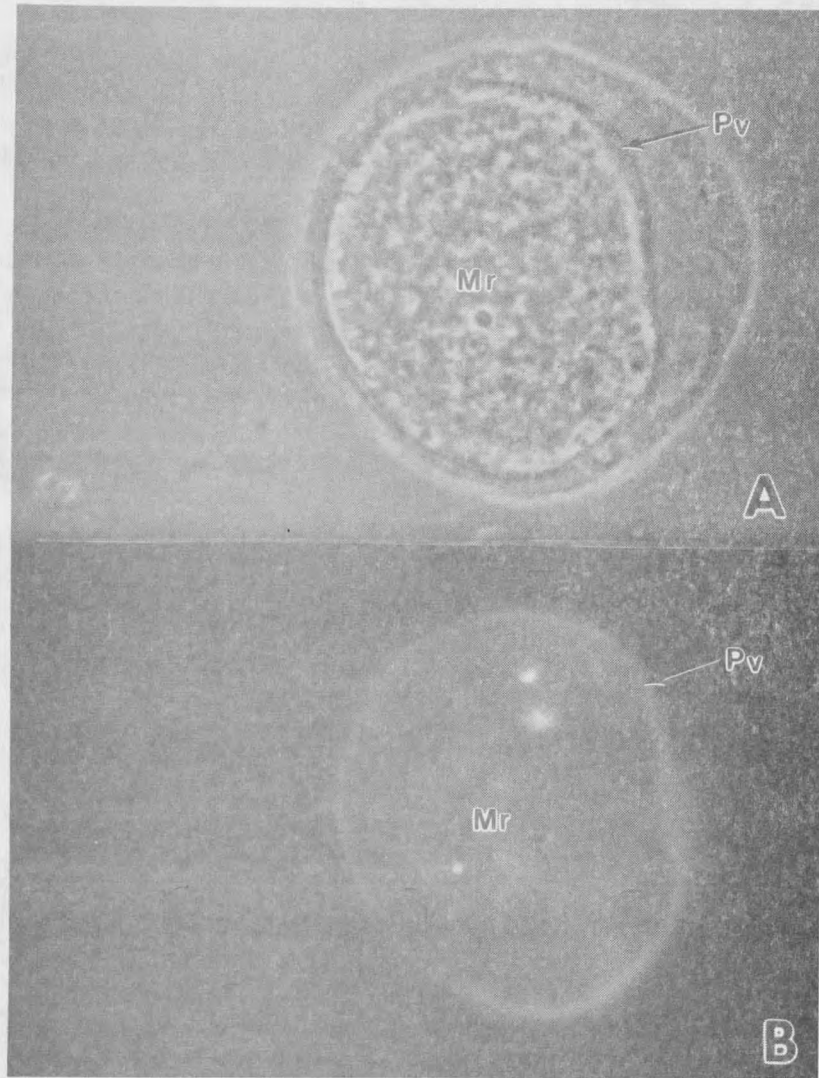


Fig. 3. Phase-contrast (A) and epifluorescence (B) photomicrographs showing reaction of EbS9 with a methanol-fixed meront of in cultured M617 cells. A. Intermediate meront (Mr), 15 days after inoculation. Note granulated material in parasitophorous vacuole (Pv). X 630. B. Same specimen as A showing intense fluorescence of granular material in parasitophorous vacuole and moderate fluorescence of meront of E. bovis. X 630.

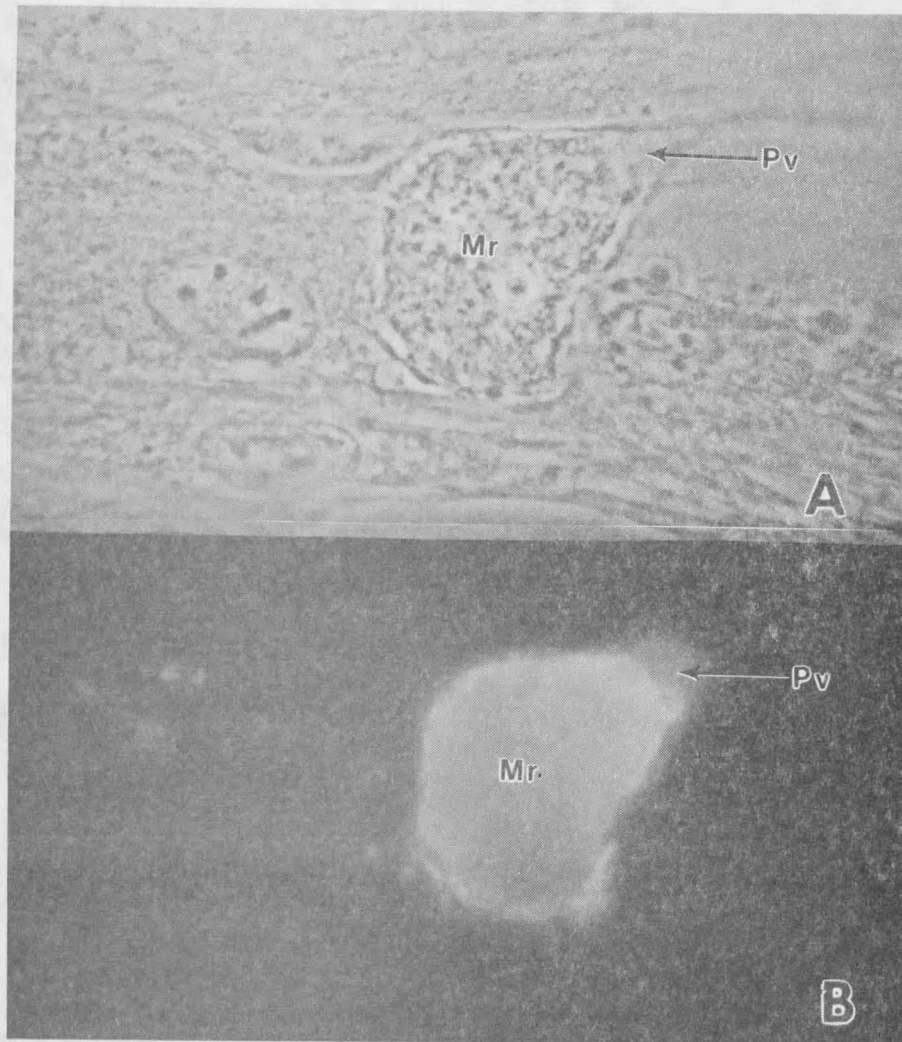


Fig. 4. A. Phase-contrast photomicrograph of meront. X 630. B. IFA Photomicrograph of the same specimen in A showing fluorescence of both meront (Mr) and parasitophorous vacuole (Pv). X 630. Treatment: methanol-fixed, EbS15, fluorescein-conjugated goat antimouse IgG.

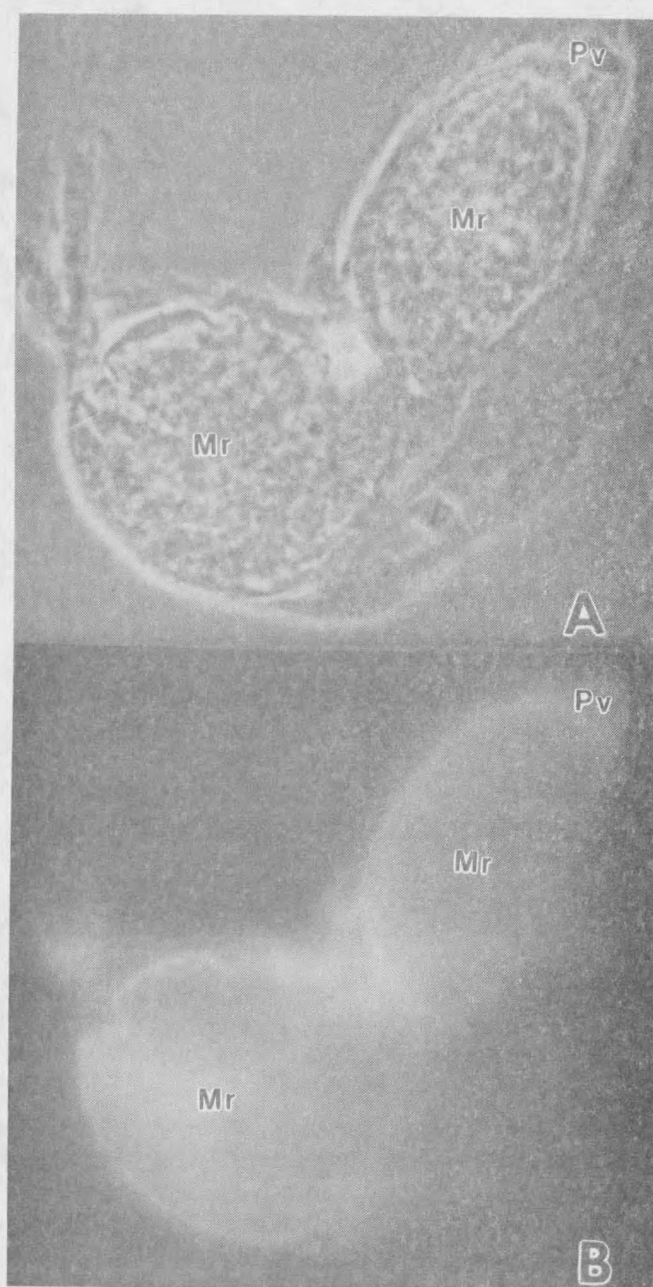


Fig. 5. A. Phase-contrast photomicrograph of two meronts (Mr). X 630. B. IFA photomicrograph of same specimen as A showing fluorescence of meronts and of the parasitophorous vacuole (Pv). Treatment: methanol-fixed, EbS15, fluorescein-conjugated goat antimouse IgG. X 630.

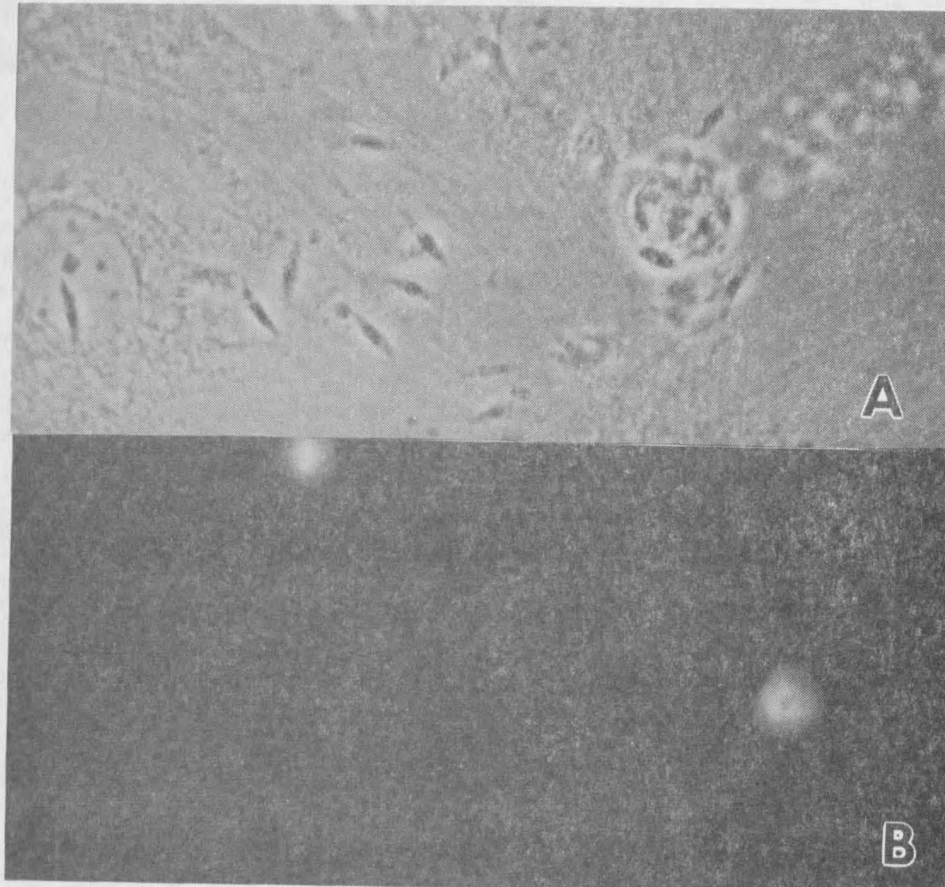


Fig. 6. Phase-contrast (A) and epifluorescence (B) photomicrographs showing reaction of EbS9 with methanol-fixed merozoites in M617 cells.
A. Extracellular first-generation merozoites 15 days after inoculation. X 630.
B. Same specimen as A, showing negative fluorescence of EbS9 with merozoites. X 630.

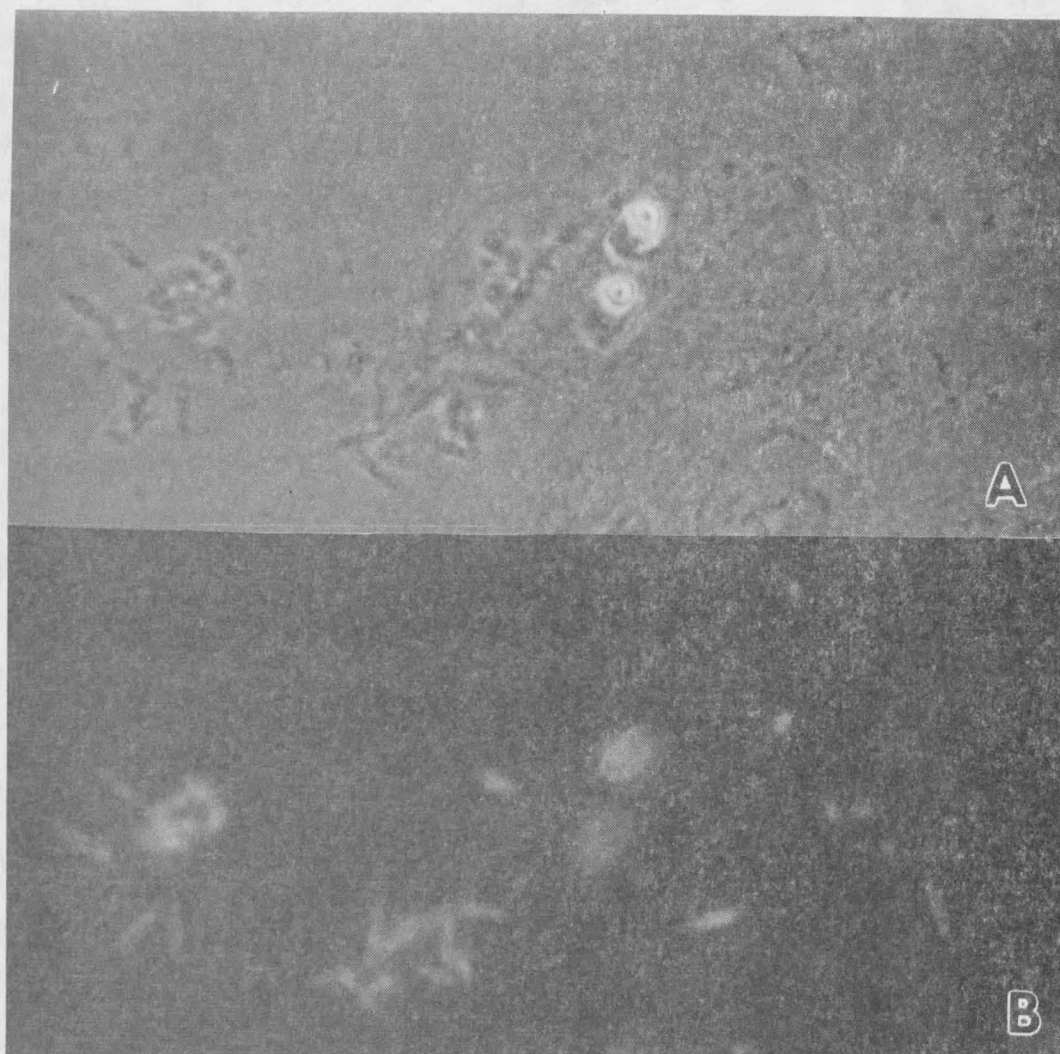


Fig. 7. A. Phase-contrast photomicrograph of two sporozoites and several merozoites in cultured M617 cells. X 630. B. Photomicrograph of IFA of the same specimen in A showing fluorescence of sporozoites and merozoites. Treatment: methanol-fixed, EbS15, fluorescein-conjugated goat antimouse IgG. X 630.

Comparison of Embedded Materials and Techniques

Although specimens embedded in Spurr's or LR White showed good preservation of antigenicity, there was considerable difference in the preservation of parasite and host cell ultrastructure. Specimens embedded in Spurr's exhibited normal ultrastructure (Figs. 8-29), whereas those embedded in LR White showed poor ultrastructural preservation with damaged organelles as well as numerous holes in the sections which may have resulted from poor infiltration and polymerization of LR White. Even though embedding in Spurr's gave superior results compared to LR White, it was somewhat more troublesome due to the longer incubation times required between antibody treatments (150 min vs 75 min with LR White) and to more rinses in BSA being required in order to reduce nonspecific binding (4 rinses vs 2 rinses for LR White).

The pre-embedding technique (Figs. 8-11) was used to localize EbS9 and EbS15 antibody receptors on the surface of parasites, whereas a post-embedding technique (Figs. 12-29) was used to localize their intracellular receptors or precursors. Pre-embedded specimens consisted of pellets of parasite-infected M617 cells that were chemically fixed, treated with MABs and colloidal gold-conjugated antibodies and post-fixed prior to being embedded in Spurr's. Ultrathin sections were then stained with uranyl acetate and lead citrate and examined with

transmission electron microscopy. In contrast, the post-embedding technique involved treating sections (on nickel grids) of parasite-infected M617 cells that had been previously fixed and embedded in Spurr's. This technique was necessary in order to visualize the intracellular localization of EbS9 and EbS15 receptors because antibodies do not cross cellular membranes.

Immunoelectron Microscopy of Pre-embedded Parasites

In pre-embedded sporozoites and merozoites of E. bovis that had been purified via passage through nylon wool, EbS9 reacted only against the surface of sporozoites (Figs. 8-11). Interestingly, sporozoites within a single preparation exhibited considerable variation in the amount of EbS9 receptors as demonstrated by the amount of colloidal gold bound to their surface (compare Figs. 8-11). Some sporozoites showed relative high colloidal-gold binding (Figs. 8-9), whereas others exhibited low to intermediate binding (Figs. 10-11), and a few showed little or no binding (data not shown). EbS15 did not react with sporozoites or merozoites, nor did EbS9 react with first-generation merozoites.

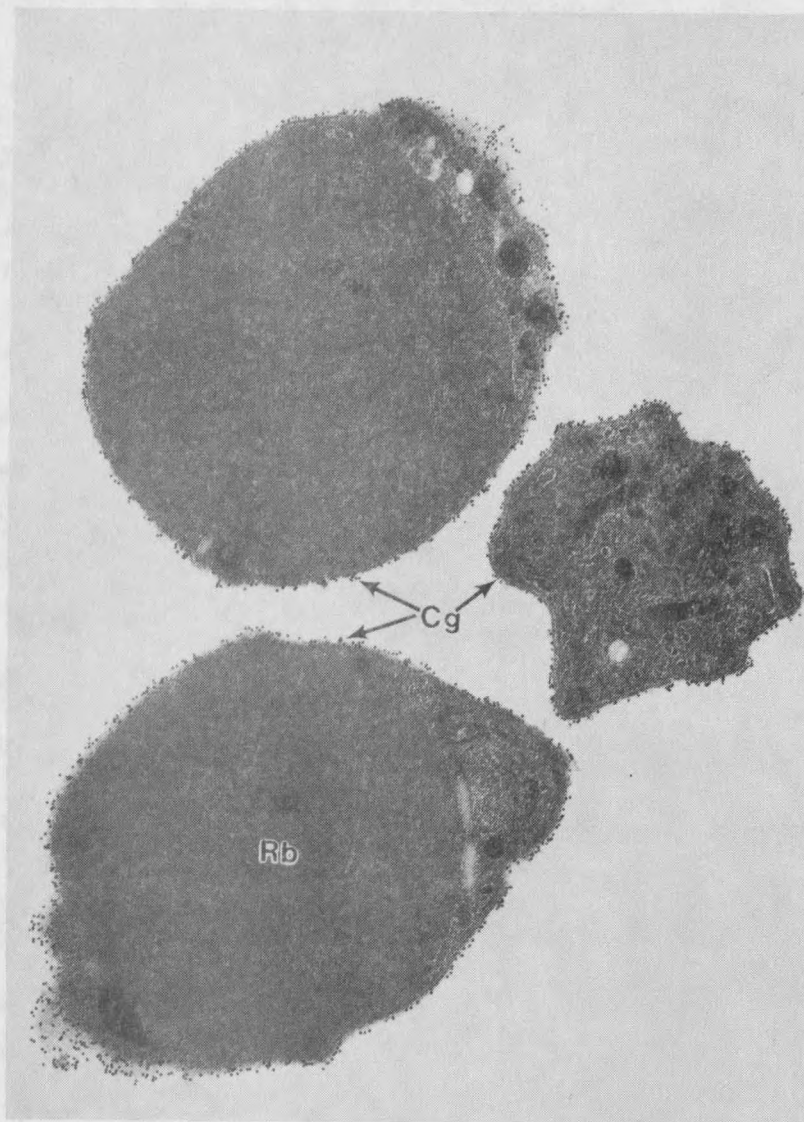


Fig. 8. Pre-embedded sporozoites treated with Ebs9 showing numerous colloidal gold particles (Cg) bound to the plasmalemma of sporozoites. X 20,000.



Fig. 9. Pre-embedded sporozoite treated with EbS9 showing numerous colloidal gold particles on its surface. X 32,000.

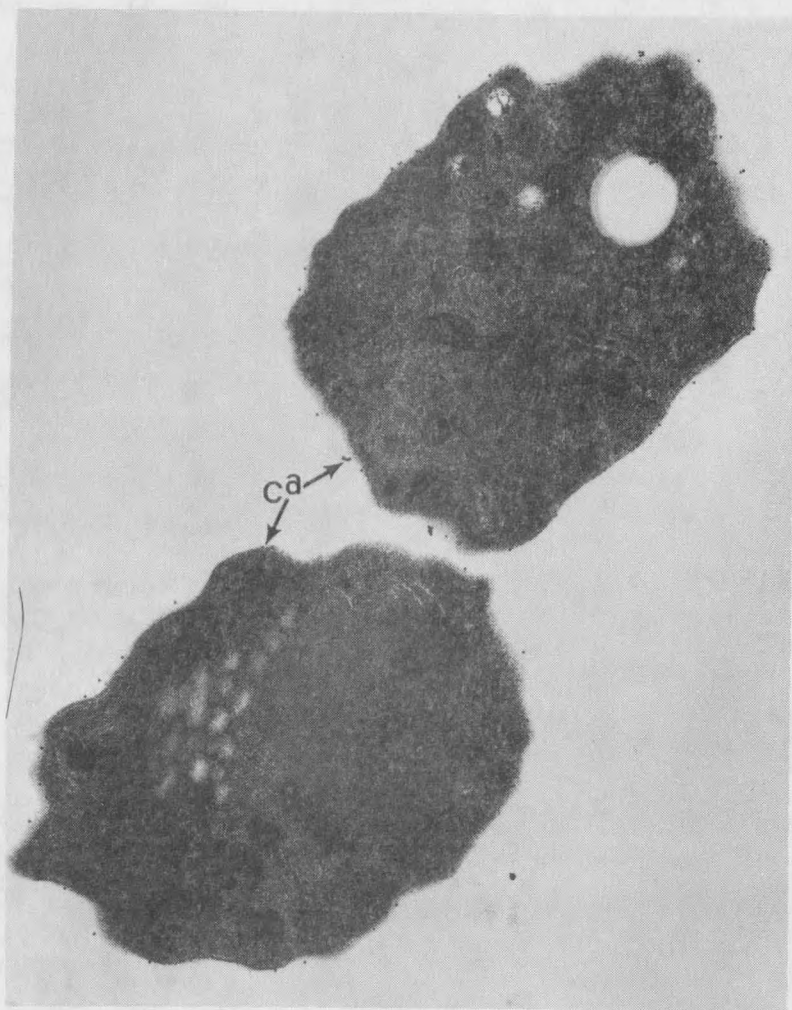


Fig. 10. Sporozoites treated with EbS9 showing intermediate number of colloidal gold (Cg) particles on their surface. X 20.000.

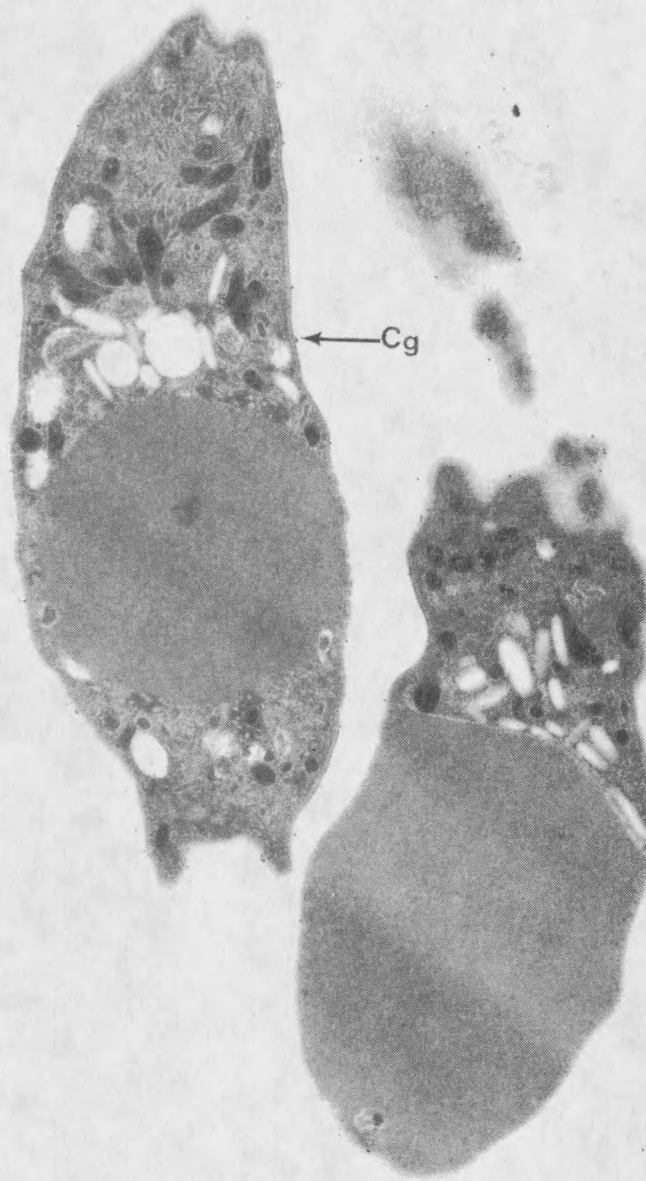


Fig. 11. Pre-embedded sporozoites treated with EbS9 showing low binding of colloidal gold (Cg) particles to their plasmalemmae. X 16,600.

Immunoelectron Microscopy of Post-embedded Parasites

Ultrastructurally, sporozoites and merozoites of E. bovis contain a nucleus, nucleolus, one or more mitochondria, endoplasmic reticulum, Golgi complex, ribosomes, polysomes, a pellicle consisting of a plasmalemma and a double inner membrane complex, 22 subpellicular microtubules, a posterior and sometimes an anterior refractile body (these are absent in merozoites), amylopectin granules, electron-dense granules, and an apical complex (Fig. 12). The apical complex, which is believed to be used in penetration of cells of the host, consists of conoid, two apical rings, two polar rings, micronemes and rhoptries (Fig. 12).

At various intervals after sporozoite inoculation, the cultured M617 cells were harvested and processed according to the post-embedding procedures described (materials and methods). Ultrathin sections of E. bovis-infected M617 cells on nickel grids were treated with EbS9 or EbS15 followed by goat anti-mouse IgG colloidal-gold antibody. In post-embedded sporozoites treated with EbS9, colloidal gold particles localized internally within rhoptries as well as near the cytoplasmic surface of rhoptries, especially in the neck region of the rhoptries (Figs. 13-16). The colloidal gold particles were usually within or on the cytoplasmic surface of empty rhoptries, which evidently had discharged their contents during

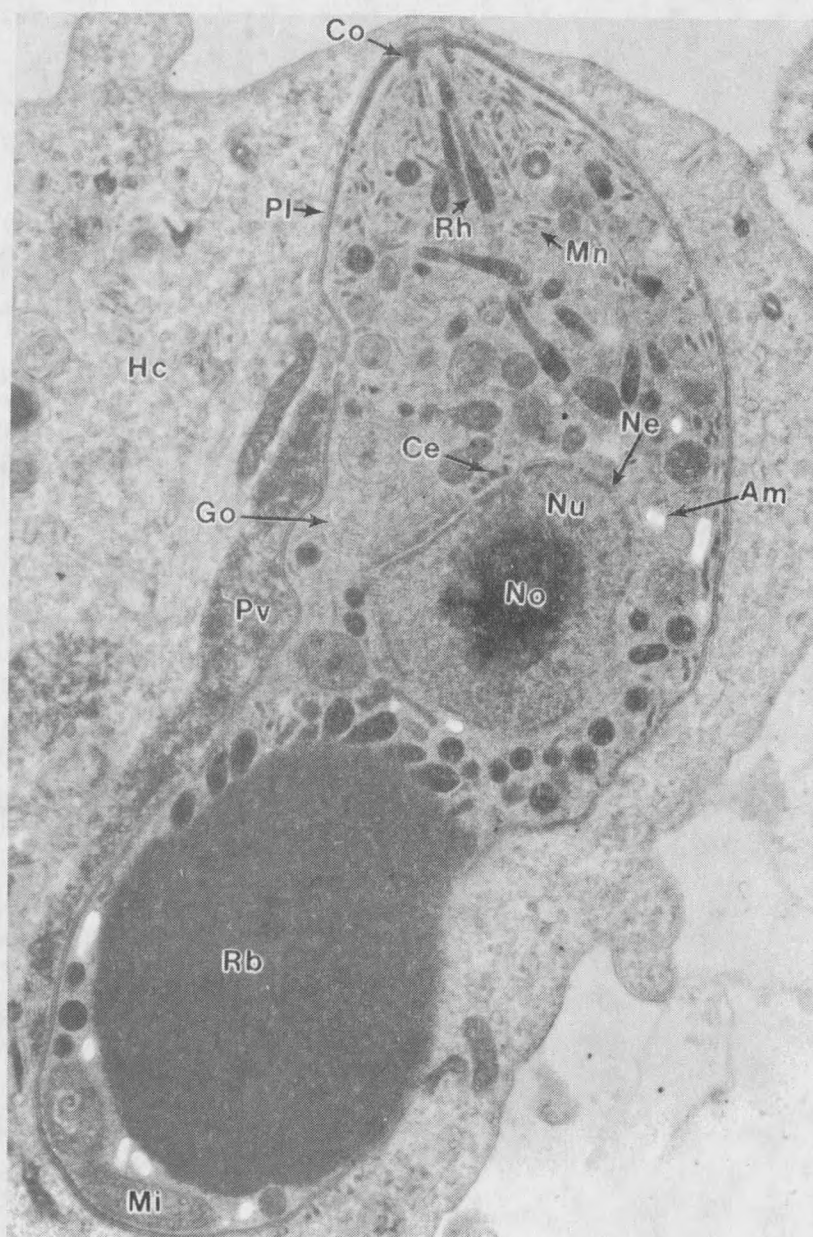


Fig. 12. Sporozoite of *E. bovis* showing typical fine structural features including conoid (Co), rhoptry (Rh), microneme (Mn), Golgi complex (Go), mitochondrion (Mi), centrioles (Ce), amylopectin (A), refractile body (Rb), nucleus (Nu), nucleolus (No), nuclear envelope (Ne), parasitophorous vacuole (Pv), plasmalemma of sporozoite (Pl) and host cell cytoplasm (Hc) Post-embedded. X 15,000.

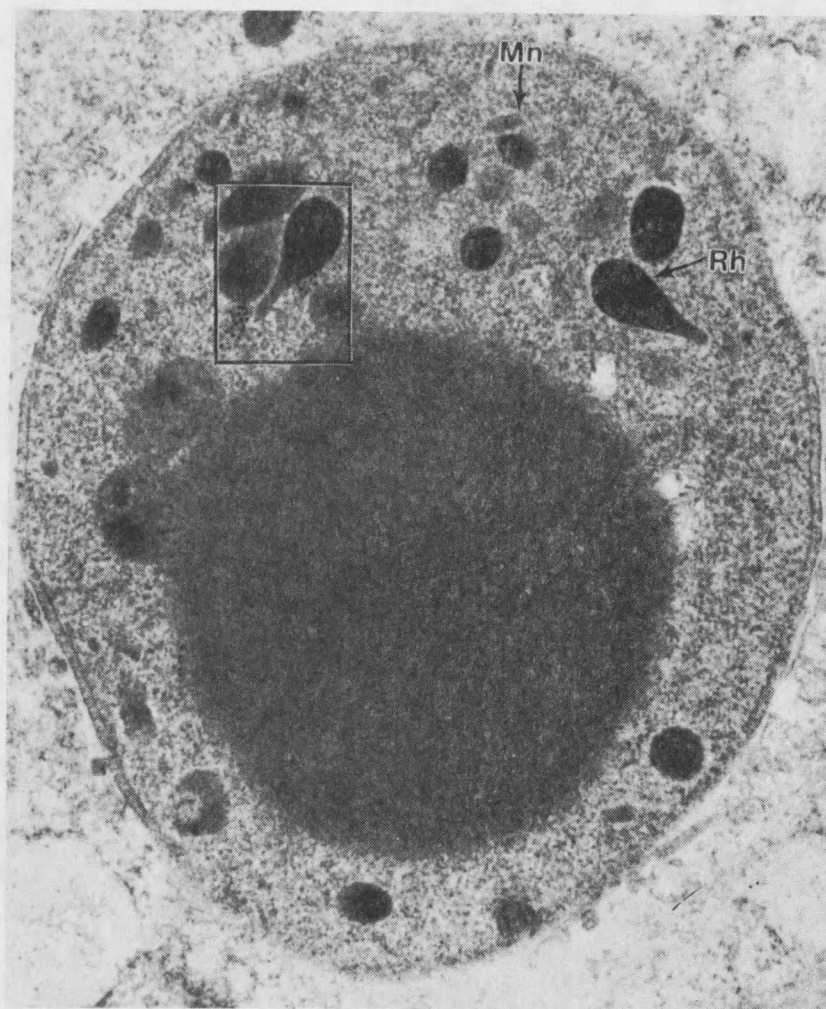


Fig. 13. Cross-section of post-embedded sporozoite treated with EbS9 shows colloidal gold particles closely associated with surface of rhoptries. X 32,000.

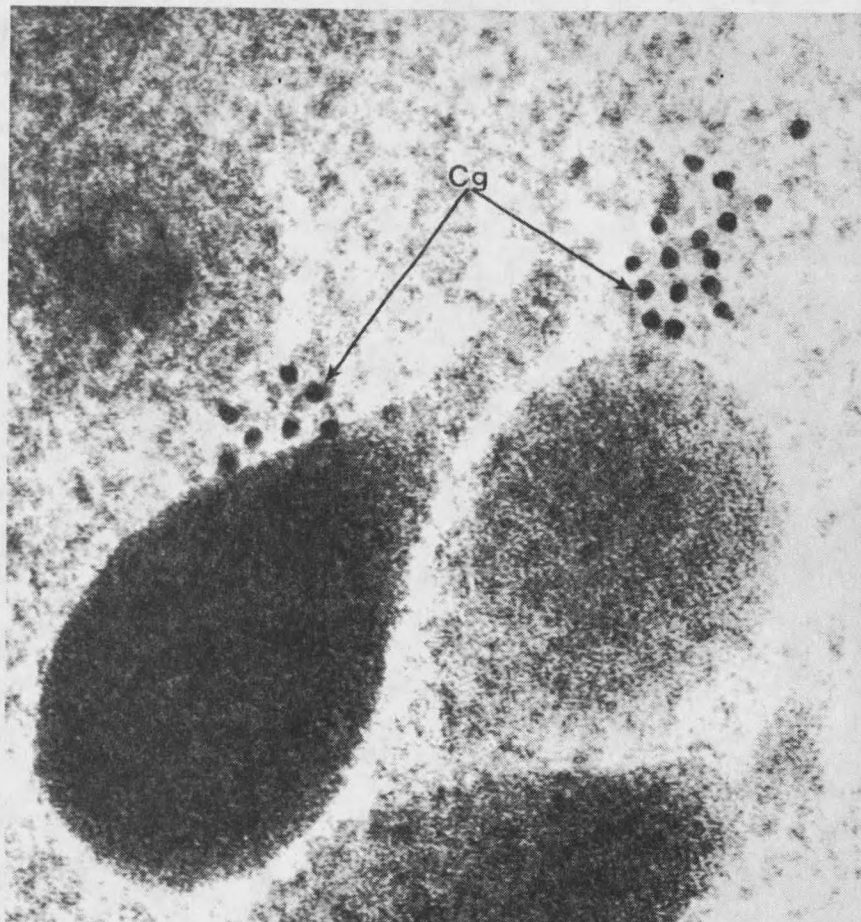


Fig. 14. Higher magnification of Fig. 13 showing colloidal gold particles closely associated with the rhoptries. X 19,800.



Fig. 15. A. Post-embedded sporozoite within cultured bovine monocyte treated with EbS9. X 13,200. B. High magnification of square shows colloid gold bound to the neck of a rhoptry. X 80,000.

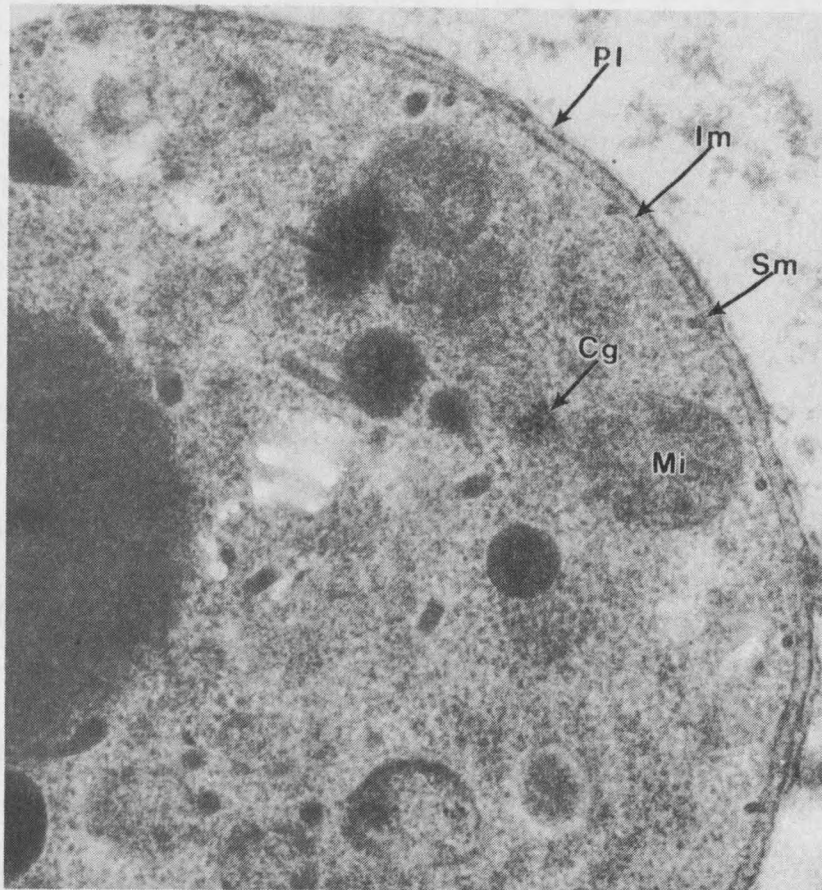


Fig. 16. Cross-section of post-embedded sporozoite treated with EbS9. Note colloidal gold particles at the neck of a rhoptry. Plasma-lemma of sporozoite (Pl); inner membrane complex (Im); subpellicular microtubule (Sm). X 54,000.

sporozoite penetration of the M617 cells (Fig. 17). Some colloidal-gold binding was found at the junction between the rhoptry-microneme complex and the nuclear envelope (Fig. 18).

In early and intermediate meronts treated with Ebs9, the colloidal gold particles occurred within the parasitophorous vacuole as well as on the plasmalemma of the meront (Figs. 19-20). Little or no colloidal gold particles were present within the meront. Occasionally, a few gold particles were found within budding and fully-formed merozoites, but no particles were found within nor on extracellular merozoites.

In post-embedded sporozoites, meronts and merozoites treated with Ebs15, the colloidal gold particles localized on amylopectin granules (Figs. 22-29). At 1 hr after inoculation, most of the amylopectin granules were located in the mid-region of the sporozoite, near the nucleus and between the anterior and posterior refractile bodies; a few amylopectin granules were located anterior to the anterior refractile body and posterior to the posterior refractile body (Figs. 23-25). Also at 1 hr after inoculation, some sporozoites had relatively few amylopectin granules indicating that amylopectin may have been expended during sporozoite motility and penetration of M617 cells (Fig. 26). At 1 to 15 days after inoculation, considerably fewer colloidal gold particles

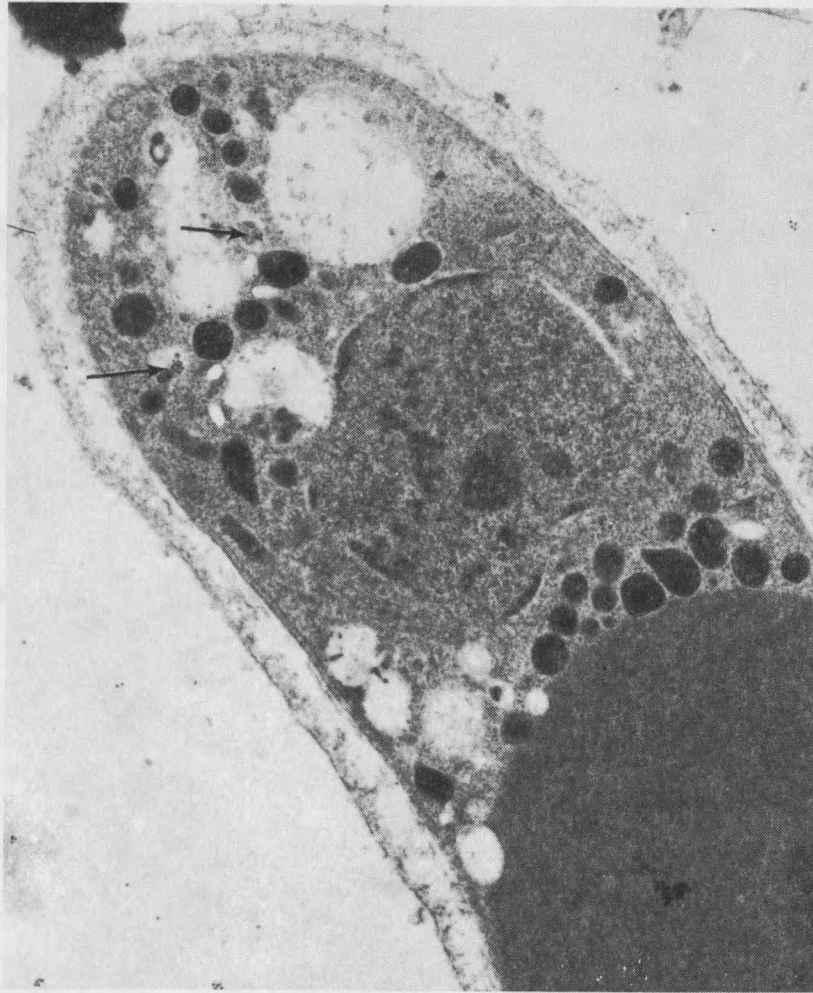


Fig. 17. Post-embedded sporozoite treated with EbS9 showing colloidal gold particles in partially empty rhoptries (arrows). X 19,800.

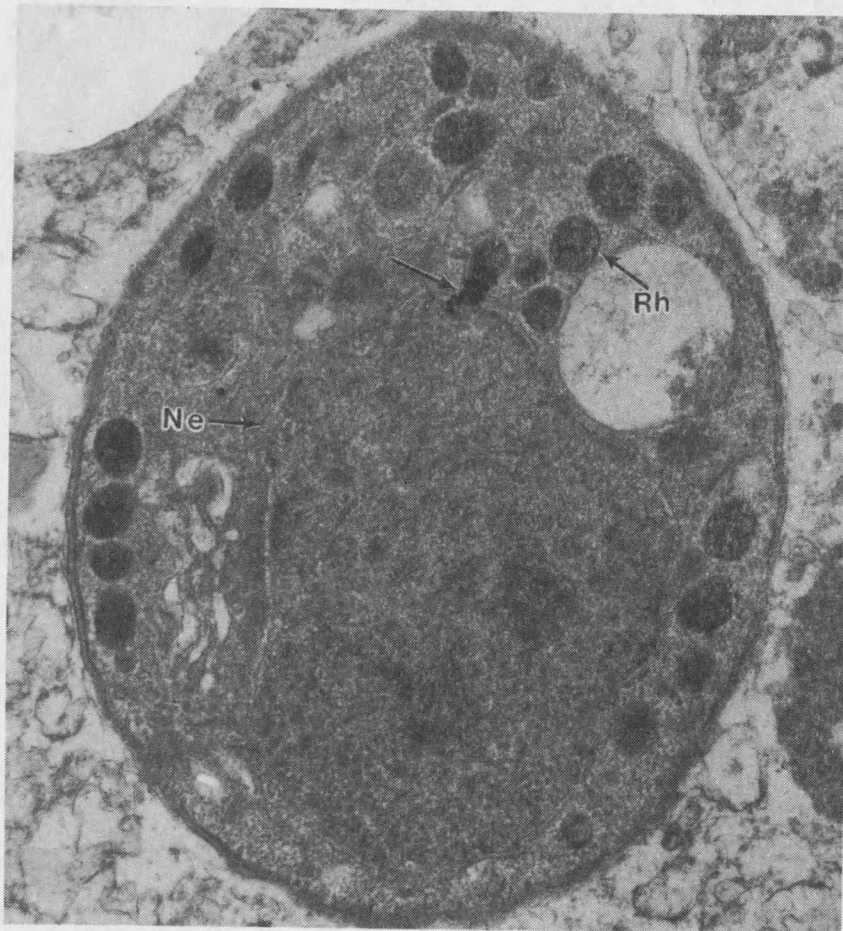


Fig. 18. Ultrastructural localization of EbS9 receptors by colloidal gold particles (arrow) between rhoptry (Rh) and the nuclear envelope (Ne). Treatment: post-embedded, EbS9, gold-conjugated goat antimouse IgG. X 26,000.

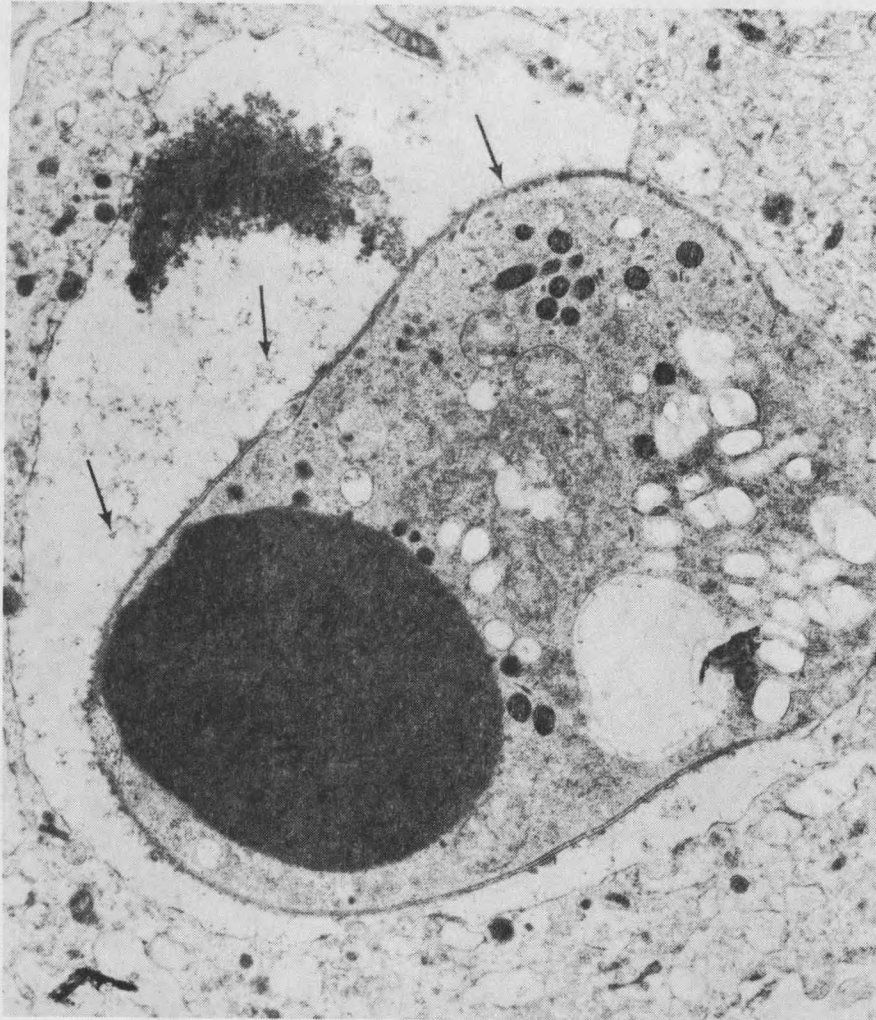


Fig. 19. Early meront at 10 days after sporozoite inoculation, colloidal gold particles (arrow) show EbS9 receptors on the edge of the meront (Mr) and in the parasitophorous vacuole (Pv). Post-embedded and treated with EbS9. X 13,200.

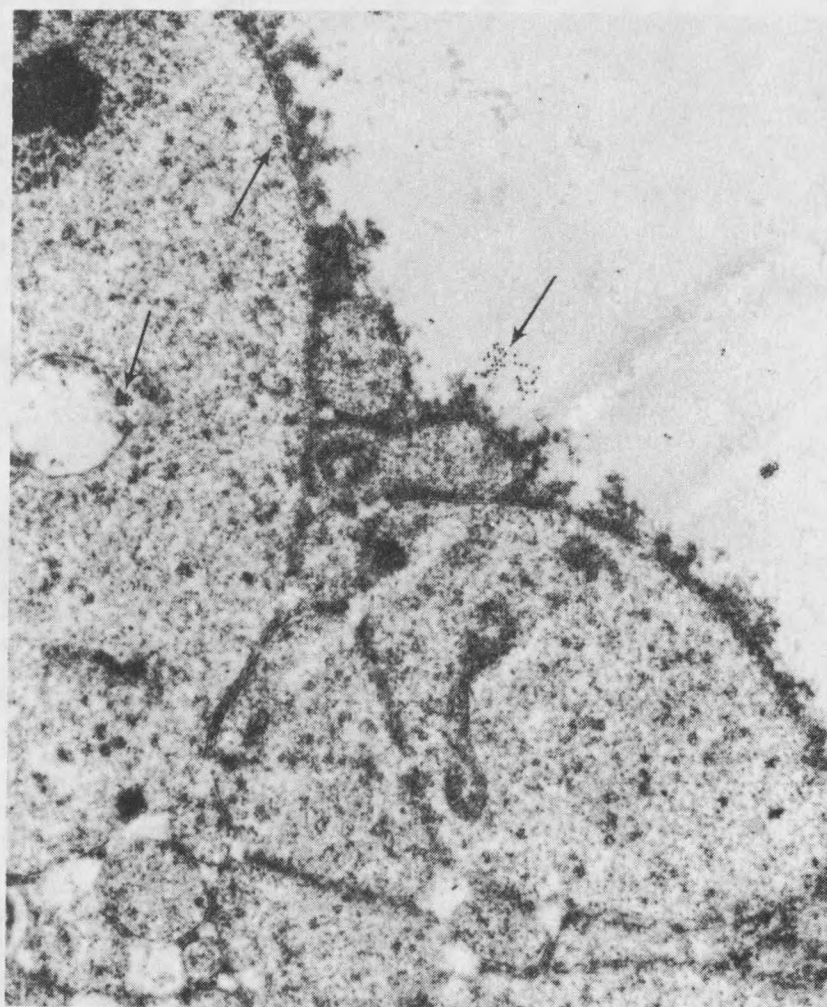


Fig. 20. High magnification of portion of first-generation meront showing EbS9 receptors on its surface and in its cytoplasm as well as in the parasitophorous vacuole (Pv). Post-embedded and treated with EbS9. X 19,800.



Fig. 21. Portion of a meront with budding merozoites. Note that the merozoites are still attached to residual cytoplasm. a few colloidal gold particles (arrow) are present inside the merozoites. Post-embedded and treated with Ebs9. X 16,600.

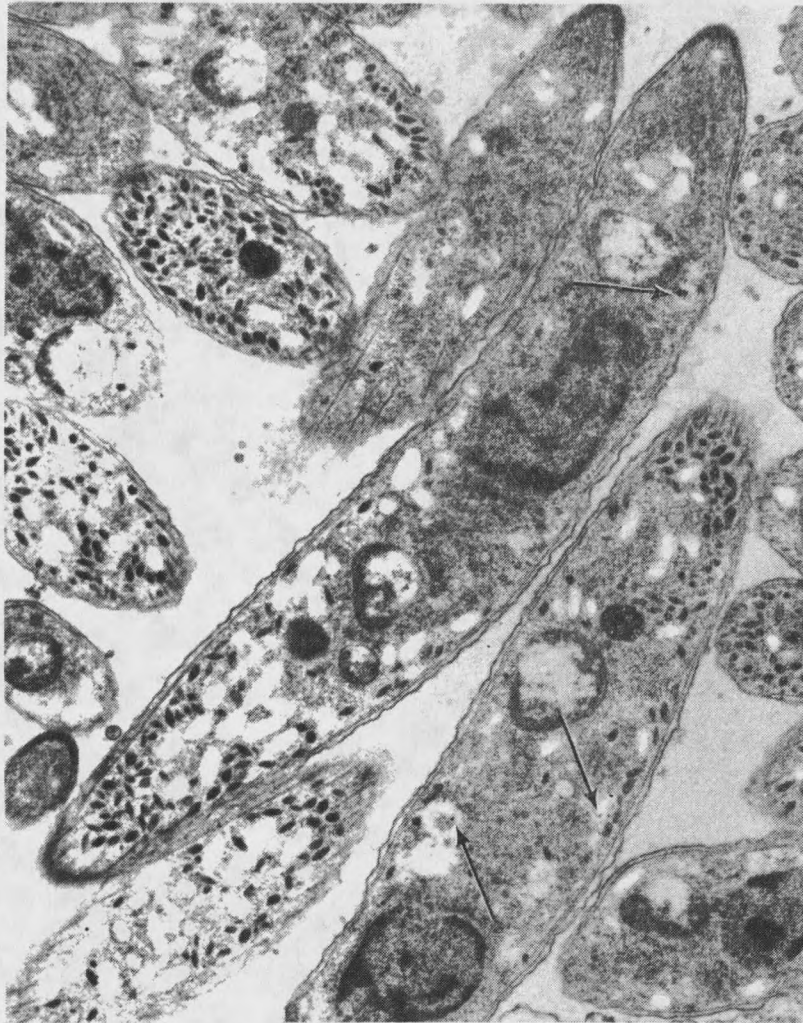


Fig. 22. Intracellular merozoites showing few colloidal gold particles inside the merozoite (arrow). Treatment: post-embedded and treated with EbS9. X 17,200.

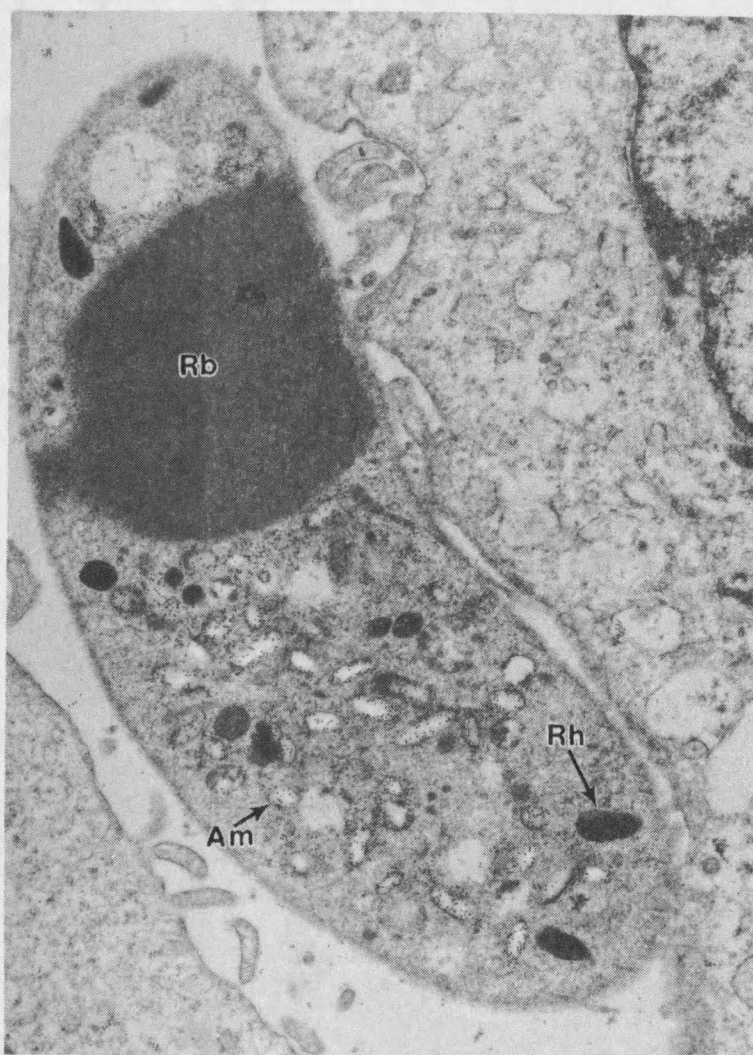


Fig. 23. Extracellular sporozoite at 1 hr after inoculation showing numerous colloidal gold particles bound to amylopectin (Am). Rb, refractile body; Rh, rhoptry. Post-embedded and treated with Ebs15. X 16,600.

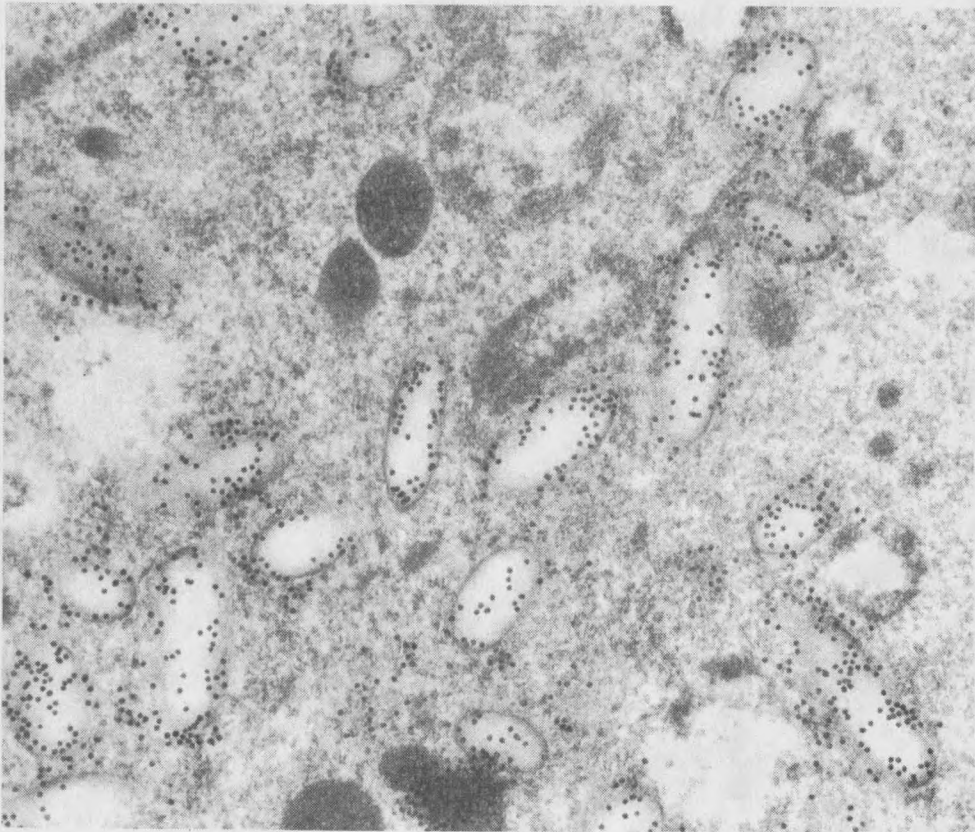


Fig. 24. Higher magnification of portion of Fig. 23 showing colloidal gold particles located at margin of amylopectin granules. X 52,000.

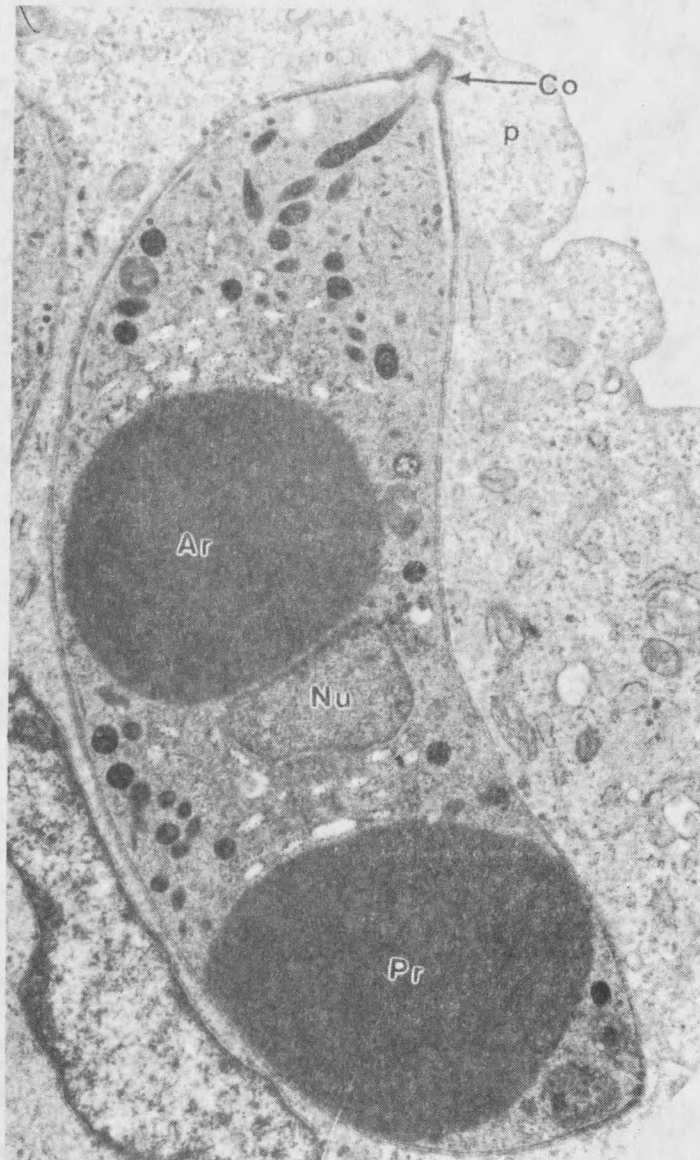


Fig. 25. Post-embedded sporozoite treated with EbS15 showing collidal gold labeling of amylopectin. Note that amylopectin is distributed in front of anterior refractile body (Ar), between the nucleus (Nu) and posterior refractile body (Pr), and behind the posterior refractile body. X 15,000.

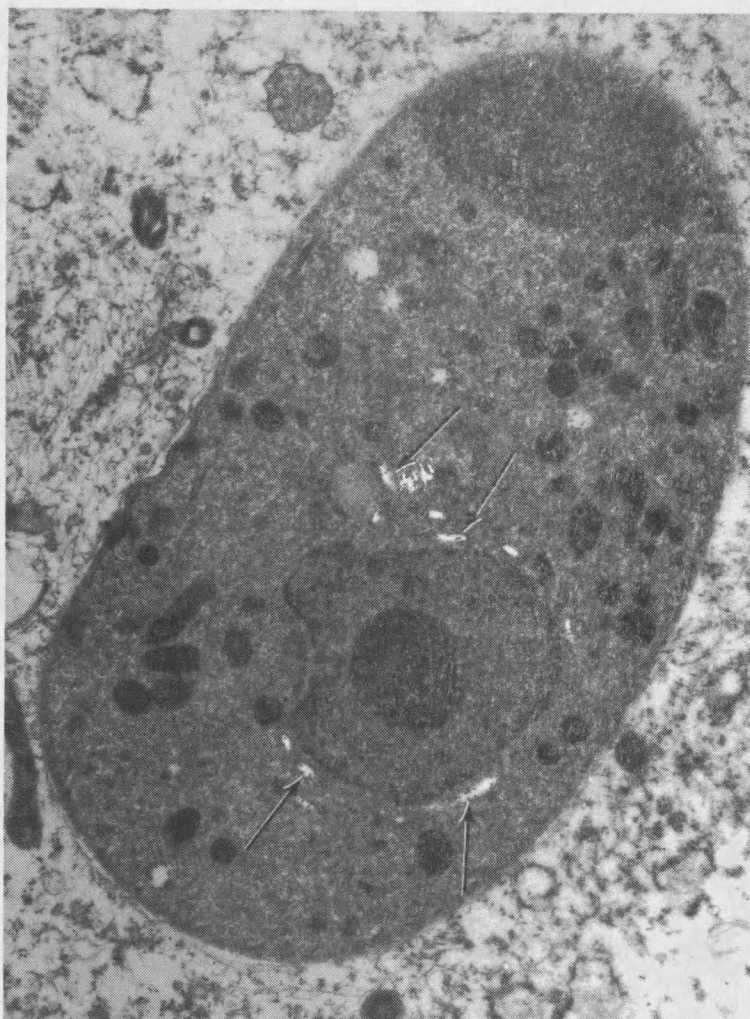


Fig. 26. Post-embedded sporozoite treated with EbS15 showing colloidal gold labeling (arrow) of amylopectin. Note that much of the amylopectin has been depleted. 1 hr after inoculation. X 20,000.

were associated with amylopectin granules in intermediate meronts and fully-formed meronts and merozoites (Figs. 27-29). Colloidal gold particles were also found in the parasitophorous vacuole and in association with membranous material in the parasitophorous vacuole surrounding intermediate and mature meronts (Figs. 28,29).

High magnification electron micrographs revealed that the colloidal gold particles were closely associated with the moderately electron-dense material at the surface of the amylopectin granules rather than the interior of the granules (Fig. 24).

Immunodetection of Sporozoite and Merozoite Antigens

SDS-polyacrylamide gel electrophoresis of non-reduced, solubilized sporozoites and merozoites showed polypeptides ranging in molecular weight from approximately 15,000 to over 200,000 and above 20,000 to over 200,000, respectively (data not shown). Western blot analysis revealed that EbS15 reacted with three sporozoite polypeptides with relative molecular weights of 75, 103 and 140 kD, and with four merozoite polypeptides of 43, 70, 105 and 120 kD (Fig. 30). Interestingly, EbS15 also reacted with phosphorylase which was one of the molecular weight standards; it has a molecular weight of 97.9 kD.

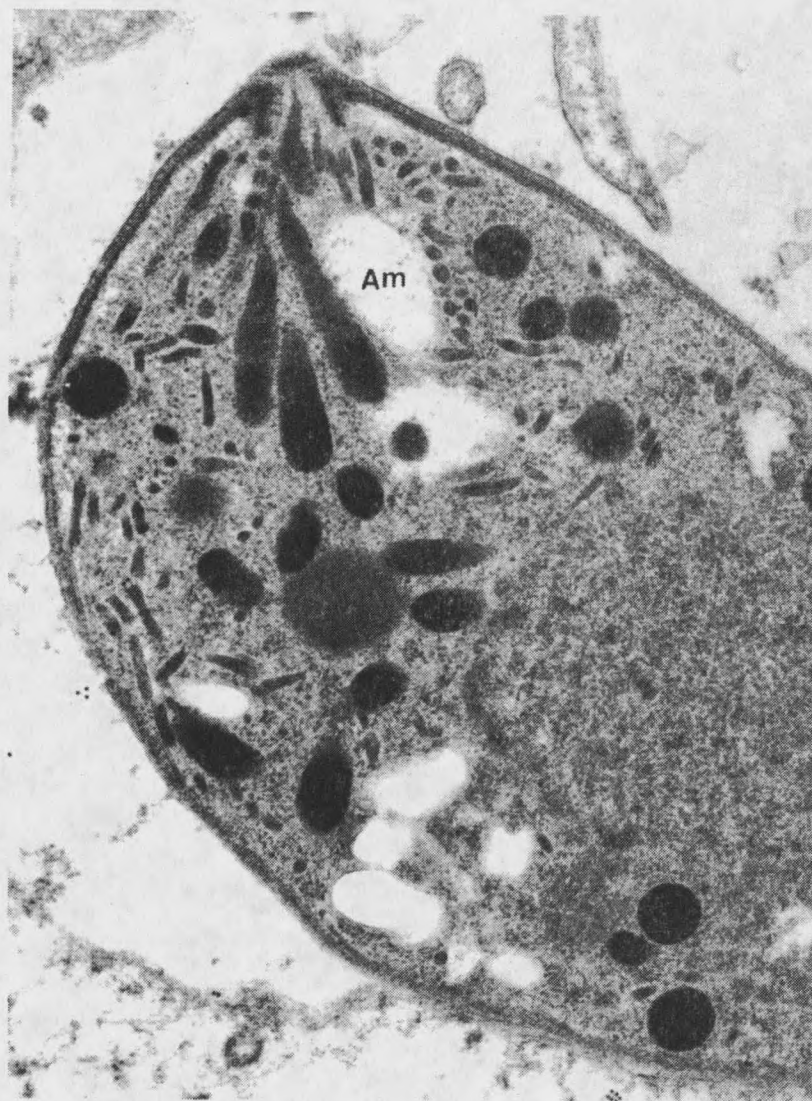


Fig. 27. Sporozoite 1 day after inoculation showing amylopectin which did not react with EbS15. Post-embedded, treated with EbS15. X 30,000.



Fig. 28. Portion of post-embedded meront treated with EbS15. Colloidal gold particles appear to be associated with amylopectin (arrow) and lipid bodies (double arrow) and in the parasitophorous vacuole. X 16,600.

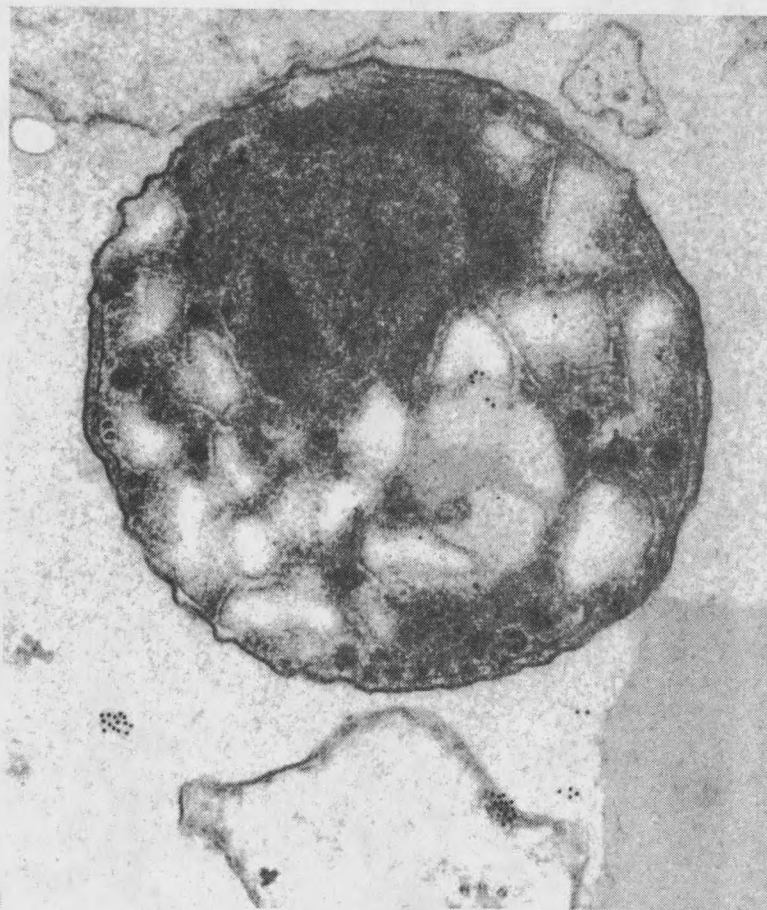


Fig. 29. High magnification of cross-section of post-embedded merozoite treated with EbS15 showing colloidal gold particles located at the surface of amylopectin and within the parasitophorous vacuole. X 200,000.

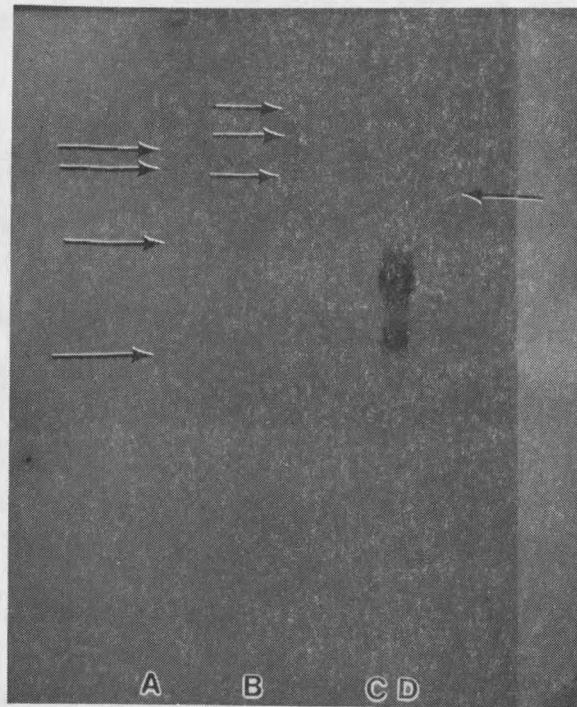


Fig.30

Western blot analysis using EbS15 reacted against *E. bovis* sporozoite and merozoite polypeptides which were transferred from 10% SDS-polyacrylamide gel to nitrocellulose paper. Lane A, merozoite proteins; lane B, sporozoite proteins; lane C, positive control (MAb 15D6 against VSV protein, unpublished data) lane D, molecular weight standard. Note that lane A has 4 bands (arrows), lane B has 3 bands (arrows) and 1 band shows in lane D (arrow) which is 97.9 kD phosphorylase.

DISCUSSION

In general, Eimeria spp use two ways of entering host cells. 1. Active Penetration: This mechanism of cell penetration by sporozoites and merozoites is preceded by at least two events, motility of the parasites including flexing and gliding, and the formation of an anterior stylet-like protuberance, the extended conoid, which can be thrust forward or retracted. Entrance is usually active and rapid (within seconds in many instances) and begins when the anterior protuberance comes in contact with the host cell plasmalemma which invaginates due to the advancing parasite to form a cavity. The host cell plasmalemma at the site of entry forms a ring causing a constriction of the parasite as it enters. When penetration is completed, the parasite is enclosed within a vacuole (called the parasitophorous vacuole) in the host cell. This type of penetration is an active process initiated and carried out by the parasite (65-69). 2. Passive Ingestion (Phagocytosis): Some Eimeria spp. appear to enter host cells by a passive process by being phagocytosed by the host cell (70,71). Some researchers have suggested that sporozoites of E. tenella and E. acervulina (avian coccidia) pass through the intestinal epithelium into the lamina propria where

they are phagocytosed by macrophages which transport them to the glandular epithelium for further development (9,72,73). Several studies have shown, however, that treatment of established cell lines with anti-phagocytic agents (reagents blocking glycolysis, thus reducing phagocytosis) does not affect the ability of the sporozoites of these Eimeria spp. to enter host cells (74,75).

In the case of E. bovis, penetration into host cells appears to be an active process. Within the first few hr after inoculation, the sporozoites of E. bovis penetrated and exited several cells before finally remaining intracellular to undergo further development. It is believed that the Apicomplexans actively enter cells by using the organelles of their apical complex to penetrate through the plasmalemma of the host cell, but the whole process of cell penetration is still not known. A penetration-enhancing factor (PEF) has been found in tachyzoites Toxoplasma gondii (14-16) and membranous tubules or whorls secreted during penetration by Plasmodium falciparum sporozoites and merozoites and by tachyzoites of T. gondii (13,16-19) are believed to be involved in host cell penetration. Furthermore, the materials appear to be derived from the rhoptry-microneme complex. In E. magna, the rhoptries appear to secrete their contents at the anterior tip of the sporozoite during penetration of

cultured cells (11). Thus, it is likely that a similar process occurs during penetration of cells by E. bovis.

In the IFA assays (present study), monoclonal antibody EbS9 reacted with the anterior one third of methanol-fixed E. bovis sporozoites. In previous studies, EbS9 was shown to react in Western blots with a 20 kD immunodominant surface antigen (called P20) of E. bovis sporozoites and to inhibit penetration of E. bovis sporozoites into cultured cells (42,43). In the present study, immunoelectron microscopy was used to determine the ultrastructural location of P20. The immunogold technique revealed that P20 or P20 precursors were located primarily on the outer surface of the plasmalemma and in the rhoptry-microneme complex of E. bovis sporozoites. P20 was also found associated with empty rhoptries of sporozoites which evidently discharged their contents during host cell penetration.

It was somewhat disappointing to find relatively little P20 (as determined by EbS9 colloidal gold labeling) in the apical complex of E. bovis sporozoites. As the parasite underwent merogony, immunoelectron microscopy showed that P20 was located on the meront plasmalemma and within the parasitophorous vacuole. Evidently, P20 was not expressed by mature meronts and fully-formed merozoites. Some immunogold binding occurred on intracellular type 1 and 2 first-generation merozoites

(76). The gold particles may have represented non-specific binding to the merozoites. However, no immunogold binding occurred with extracellular merozoites. This indicates that some P20 may have remained within merozoites still within meronts which was lost once merozoites became extracellular.

P20 also appeared to be localized between sporozoite rhoptries and the nuclear envelope. In P. berghei sporozoites and merozoites, membranous whorls in the nuclear envelope appear to be part of a continuous endomembrane system, involving the nuclear envelope, the rough endoplasmic reticulum, Golgi complex and the rhoptry-microneme complex in the same sense that the nuclear envelope and rough endoplasmic reticulum are components of a continuous endomembrane secretion system common to higher eukaryotic cells. Rhoptries of T. gondii tachyzoites are known to be derived from the Golgi complex which itself is directly formed from the nuclear envelope (32,33). Thus, it appears that P20 may be synthesized within nuclear envelope, pass through the endoplasmic reticulum, the Golgi complex, microneme, rhoptry, and then secreted to the surface of the sporozoite (plasmalemma) via the necks of rhoptries.

Immunoelectron microscopy involving the intracellular localization of receptors for EbS15, showed that the antigen (called PX) against which this MAb reacts was

associated with amylopectin granules of E. bovis. Amylopectin granules of sporozoites contained more PX than did those of intermediate and mature meronts and merozoites. During the life cycle of E. tenella, amylopectin repeatedly appears and disappears and it is the only carbohydrate found in large amounts in Eimeria spp. (77). Biochemistry studies of amylopectin granules have shown that they consist of glucose polymers only. Amylopectin granules appear similar ultrastructurally whether in plants or protozoa (78). In electron micrographs of E. bovis sporozoites, amylopectin appears as oval granules with a uniform size of 0.5 X 0.7 μm and is distributed in 3 regions: (a) in front of the anterior refractile body, (b) around the nucleus and (c) behind the posterior refractile body.

A amylopectin granules are generally regarded as one of the energy reservoirs for coccidian parasites. In an early study, sporozoites within the lamina propria that were devoid of amylopectin were considered to lack the necessary energy to reach cells at the base of the crypts of Lieberkerühn (79,80). Amylopectin is evidently used as an energy source for excystation and subsequent penetration of host cells, but this has not yet been thoroughly elucidated (81,82).

In the present study, at 1 hr after inoculation numerous PX receptors for EbS15 were found associated with

numerous amylopectin granules which were distributed in the front of the anterior refractile body; around the nucleus and behind the posterior refractile body. At 1 day to 15 days after inoculation, however, there was a decrease in the number of amylopectin granules as well as a decrease in the number of PX receptors associated with the amylopectin granules in sporozoites. This indicates that sporozoites of E. bovis utilize the amylopectin as a major energy source to penetrate host cells and to initiate intracellular development (39). It is possible, that amylopectin and its related enzyme system are active during the first few hours after the parasites finally become intracellular. Amylopectin phosphorylase, which appears to be the major means of amylopectin utilization throughout sporulation, may also be involved during intracellular development of sporozoites. Electron microscopic studies have shown only the number of granules changes during sporulation which indicates that the amylopectin phosphorylase must attack and completely degrade each granule before moving on to the next one (10). Although the mechanism by which this is accomplished is not known, the observation that purified amylopectin granules do not serve as substrate for phosphorylase suggests that there might be some factor associated with the granules in vivo which increases their susceptibility to phosphorylase. This factor could be

responsible for the sensitive degradation of the granules, however, the mechanism by which amylopectin phosphorylase and its related factor are turned on during penetration and probably turned off after the parasite becomes intracellular remains to be determined.

In this study, relatively few PX molecules were associated with amylopectin granules of *E. bovis* meronts and merozoites. This may have been due to the fact that the meronts and merozoites were grown in an in vitro system. Although, merozoites of *E. bovis* grown in vitro are capable of penetrating cultured cells, they do not develop further. It is possible that either the amylopectin phosphorylase and/or its related factor are not formed by merozoites of *E. bovis* that develop in vitro which results in their inability to undergo further in vitro development.

In the SDS-PAGE study, the sporozoites and merozoites of *E. bovis* had polypeptides ranging in molecular weight from 15 to more than 200 kD. Western blots revealed that EbS15 reacted with 3 sporozoite polypeptides (70, 105 and 116 kD) and with 4 merozoite polypeptides (43, 70, 103 and 110 kD). These findings indicate that several polypeptides of sporozoites and merozoites of *E. bovis* contain the epitope against which EbS15 reacts. Interestingly, EbS15 reacted against the 97.9 kD molecular standard which was phosphorylase. This

indicates that PX (against which EbS15 reacts) is probably a phosphorylase enzyme with a molecular weight of 105 kD in sporozoites and 103 kD in merozoites, and may be associated with the degradation of amylopectin. Close examination of high magnification immunoelectron micrographs showed that PX was associated with the moderately electron-dense materials on the surface of amylopectin granules and not the granule itself. Thus, the moderately electron-dense material must evidently represent amylopectin phosphorylase or its related structure.

In attempts to develop a post-embedding system for immunoelectron microscopy, the present study showed that LR White and Spurr's medium provided about the same antigenic preservation. LR White embedded material exhibited excessive ultrastructural artifacts and high nonspecific background staining with colloidal gold. In contrast, Spurr-embedded specimens showed excellent ultrastructural detail and little or no nonspecific staining. I also found that Spurr-embedded materials could be post-fixed in 1% osmium tetroxide without loss of antigenicity. Instructions provided by London Resin Company stated that in order to preserve antigenicity post-fixation in osmium tetroxide should be avoided. Spurr's medium is an epoxy resin, whereas LR White is an acrylic resin. According to Hardy et al. (83) and

Moriarty and Halmi (84) better tissue preservation can be achieved with epoxy resins than with glycol methacrylate. Based on the results of earlier studies, osmium tetroxide was considered to denature many antigenic determinants and, consequently most post-embedding immunostaining was performed on aldehyde-fixed tissue without post-osmication (85,86). However, Dacheux and Dubois (87) demonstrated that some pituitary hormones could be immunostained after primary fixation in osmium tetroxide and embedding in Epon. Other workers have been able to demonstrate that not only all the currently known pituitary hormones but also several neuropeptides can be readily immunostained in tissue fixed in glutaraldehyde, post-fixed in osmium tetroxide and embedded in Epon in the conventional way (88).

SUMMARY

Eimeria bovis is the most common cause of coccidiosis in cattle, causing an annual world-wide loss of several hundred million dollars. To date, there is still no vaccine or satisfactory treatment for bovine coccidiosis. There is little information concerning the role of parasite antigens in the immune response to coccidian parasites. The objectives of this study were to 1) develop ultrastructural techniques for the intracellular localization of E. bovis antigens and 2) to determine the ultrastructural localization and fate of two antigens, P20 and PX, of E. bovis.

In order to localize internal antigens, a post-embedding technique was developed which involved applying monoclonal antibodies EbS9 and EbS15 (which react against P20 and PX, respectively) and anti-antibodies conjugated to colloidal gold directly to ultrathin section of bovine monocytes (M617 cell) infected with E. bovis. Specimens embedded in LR White exhibited excessive ultrastructural artifacts and high nonspecific staining with colloidal gold. Spurr-embedded specimens, however demonstrated excellent ultrastructural detail and little or no nonspecific staining with colloidal gold. A pre-embedding

technique was used to study the localization of P20 on the surface of E. bovis sporozoites.

Immunoelectron microscopy showed that P20 was uniformly distributed on the surface of most E. bovis sporozoites, whereas some exhibited little or no P20 on their surfaces. This indicates that P20 is expressed differently by E. bovis sporozoites which might be expected since the preparation represents heterogeneous populations of sporozoites (i.e., not a clone). P20 was gradually shed by developing meronts into the parasitophorous vacuole and was completely absent in extracellular merozoites.

Sporozoites treated with EbS9 showed apical fluorescence in IFA assay. Immunoelectron microscopy of post-embedded specimens revealed that P20 was evidently synthesized at the nuclear envelope, and then transported to the sporozoite surface by the Golgi complex, micronemes and rhoptries.

Immunoelectron microscopy using EbS15 revealed that PX was a moderately electron-dense material associated with the surface of amylopectin granules. At 1 hr after sporozoites inoculation of M617 cells, sporozoites contained numerous amylopectin granules, but few amylopectin granules were present in sporozoites at 1 day after inoculation. Western blots of solubilized parasites revealed that EbS15 reacted with four merozoite antigens

and three sporozoite antigens as well as with phosphorylase, a 97.9 kD molecular weight marker. This indicates that PX may be an amylopectin phosphorylase associated with amylopectin granules. Immunoelectron microscopy showed that EbS15 reacted weakly with amylopectin granules of intermediate meronts and fully formed merozoites, indicating that little or no phosphorylase was associated with their amylopectin granules or that phosphorylase associated with meront and merozoite amylopectin contained little of the epitope recognized by EbS15.

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