Production of Monoclonal Antibodies Specific for the Microgametocytes of Eimeria tenella

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PRODUCTION OF MONOCLONAL ANTIBODIES
SPECIFIC FOR THE MICROGAMETOCYTES
OF Eimeria tenella

by

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DEDICATION

This dissertation, and all it signifies, is dedicated to my beloved parents, my late father Fred, and my mother, Gertrude, who made all my aspirations attainable.

Marc A. Laxer
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ABSTRACT

Production of Monoclonal Antibodies Specific for the Microgametocytes of *Eimeria tenella*

by

Marc A. Laxer, Doctor of Philosophy
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Department: Biology

The objective of this study was to produce a monoclonal antibody specific for the microgametocytes of *Eimeria tenella*, examine the site and stage specificity of the antibody, and investigate the immunopotency of the antibody. BALB/c mice were immunized with antigen containing *Eimeria tenella* microgametocytes isolated from *in vitro* systems. After three intraperitoneal immunizations with the antigen and one booster immunization administered by tail vein injection, the mice were sacrificed and their spleen cells fused with SP2/0 mouse myeloma cells using polyethylene glycol as a fusing agent. Resultant hybridomas were screened by immunoelectrophoresis, indirect
immunofluorescent antibody assay, and immunoelectron microscopy to determine the isotype, subisotype, site and stage specificity of the antibody. Of four 96 well plates seeded with fusion products, four hybridomas were found to be producing antibody specific for the target antigen. Only the most strongly positive of these hybridomas, clone T1A3B9, was used for the study. The antibody produced by this hybridoma was found to be of subisotype IgG2a.

T1A3B9 monoclonal antibody was introduced into Eimeria tenella infected cell cultures on days four, five, and six post-infection. At seven days post-infection, oocyst production was assayed by fixing, staining, and counting the resultant oocysts. Results of the in vitro experiments showed a greater than 50% reduction in oocyst production in experimental cultures over controls. Statistical significance of the data were confirmed by a Mann-Whitney U Test. These results indicate that the monoclonal antibody was exerting an inhibitory effect on the fertilization process.

T1A3B9 monoclonal antibody was incubated with Eimeria tenella infected cecal scrapings and cell culture material, immunolabeled with colloidal gold conjugates, and observed by electron microscopy. Results showed that the antibody was binding to the microgametocytes and to no other life cycle stages of the parasite, nor was it binding to host tissue. This indicates that the antibody is stage specific. Additionally, the antibody was seen to bind only
to areas in close proximity to the budding flagella of developing microgametes, thus indicating distinct site specificity.
INTRODUCTION

Avian coccidiosis poses a significant economic threat to the American poultry industry. In 1965, the United States Department of Agriculture estimated that poultry producers lost approximately $35 million dollars to the disease, and, for the year 1985, the loss estimate has been increased to $300 million (Laxer et al, 1985). The causative agent of cecal coccidiosis, *Eimeria tenella*, is a protozoan parasite that infects the blind pouches, or ceca, of the small intestines of birds of the genus *Gallus*. Clinically, the disease produces hemorrhage, unthriftness, diarrhea, and anemia. Young birds are highly susceptible, particularly in flocks raised on the floor, and high mortality is not uncommon (Levine, 1982).

Since total eradication of avian coccidiosis is both economically and biologically impractical, efforts have been directed at controlling the disease (Fayer and Reid, 1982). These authors described several control methods that have received particular emphasis. Chemotherapy has been a widely applied technique, with the drugs Monensin (Ely Lilly) and Amprolium (Merck, Sharp & Dohme) being the most successful agents. The major problem with chemotherapy was the parasite's ability to develop resistance to the drugs. Good sanitation and management practices have been effective control measures, but continuous vigilance was essential. Nutritional supplementation has had limited
success. These authors stated that the method receiving intensive interest was immunologic control. The natural immunity to eimerian infections has been investigated by many workers. Rose et al (1984) described an array of immune responses in infected chickens, with emphasis on the humoral role of secretory IgA. Straneva (1984) examined the role of macrophages in the cellular immune response to coccidia. These studies, and similar works, established the groundwork for further research into the manipulation of immune functions to control parasitic infection.

Malarial organisms have been the primary targets of immunologic control efforts, and the techniques most often employed involved the use of monoclonal antibodies. However, there has been considerable work done with monoclonal antibodies directed against parasitic protozoa other than Plasmodium. In 1980, Sethi and co-workers described the successful production of eight lines of cloned cells producing antibodies with high specificity for Toxoplasma gondii. Araujo et al. (1980) used monoclonal antibodies to assay for antigens of T. gondii in the serum and body fluids of mice and humans. Handman et al. (1980) were able to detect and characterize the surface membrane antigens of T. gondii using monoclonal antibodies. Hauser and Remington (1981) described the effect of monoclonal antibodies on the phagocytosis and killing of T. gondii by normal macrophages.

The use of monoclonal antibodies is a well established
technique in the area of *Eimeria* research. Danforth and Augustine (1981) described the use of monoclonals in the species diagnosis of *Eimeria tenella*. Danforth (1982) further described the development of monoclonals that are specific for the pellicular and internal antigens of the sporozoite stages of *Eimeria tenella* and *Eimeria mitis*. Speer et al. (1983) described an ultrastructural study of antigenic sites on *Eimeria* oocysts, sporocysts, and sporozoites using monoclonal antibodies.

To complement these studies, the objectives of this project are 1) to produce a monoclonal antibody specific for the microgametocyte stage of *Eimeria tenella* and attempt to block the fertilization process using the antibody; 2) verification of the stage-specificity of the monoclonal antibody using immunoelectron microscopy; 3) verification of the site-specificity of the monoclonal antibody on the microgametocyte by immunoelectron microscopy; 4) verification of the immunologic effects of the monoclonal antibody by assaying its inhibitory effects on fertilization.
REVIEW OF THE LITERATURE

For the sake of clarity and organization, this section is divided into subsections. Each deals with a topic that is pertinent to the current investigation. The first section covers the life cycle of the parasite. Subsequent sections cover, in order, fertilization in general, fertilization in the Sporozoa, hybridoma technology, hybridomas in parasitology, and gold labeling methods in immunoelectron microscopy.

Life Cycle

The life cycles of eimerian parasites both in vivo and in vitro were reviewed in detail by several authors (Doran, 1970; Kelley and Youssef, 1977; Fisher et al 1979; Speer, 1979; Joyner, 1982; Levine, 1982). *Eimeria tenella* is the causative agent of cecal coccidiosis in the domestic chicken. It is generally accepted that *Eimeria* infections exhibit strict host and site specificity, although Kogut and Long (1984) presented evidence that *Eimeria*, like the related genera *Isospora*, *Sarcocystis*, *Hammondia*, *Toxoplasma*, and *Besnoitia*, were found as extraintestinal forms in the blood, liver, lungs, and hearts of chickens and turkeys. The primary site of infection of *Eimeria tenella*, however, is the cecum. The infection is acquired when sporulated oocysts are ingested by the host along with food
or water. Upon reaching the upper digestive tract, the oocyst undergoes the process of excystation. This is initially a mechanical process involving the disruption of the oocyst wall with subsequent release of four sporocysts. The sporocysts pass further down the digestive tract where they encounter bile salts and trypsin, which results in the liberation of two sporozoites from each sporocyst. The sporozoites eventually arrive in the cecae whereupon the infection is initiated.

The sporozoites invade the surface epithelium and are relocated to the crypts. It is at this point that the asexual reproductive cycle is begun. Sporozoites begin a series of multiple fissions known as schizogony with the resultant formation of a first generation schizont. When the schizont ruptures, first generation merozoites are released. These merozoites penetrate surrounding host cells and begin a second round of schizogony, forming second generation schizonts. When these rupture, the parasitized host cells are destroyed and second generation merozoites are liberated. It is during schizogony that the significant pathology of the disease occurs. There is considerable destruction and sloughing of host epithelium with formation of multiple petechiae. Ecchymotic hemorrhage is common and the accumulation of blood and cell debris forms a hard core in the cecal lumen. When second generation merozoites penetrate host cells, they begin the sexual phase of the reproductive cycle (Doran, 1970, reported the existence of a
third generation of schizogony in cell culture systems). Some of the second generation merozoites transform into macrogamonts (female analogs) and others transform into microgamonts (male analogs). Speer and Danforth (1976) reviewed the mechanisms of sexual differentiation in the eimerians. They reported that some merozoites developed into microgamonts whereas others developed into macrogamonts. In cell culture systems of Eimeria tenella, those merozoites that exhibited a positive Periodic acid-Schiff (PAS) reaction developed into macrogamonts, while merozoites that showed a PAS-negative reaction developed into microgamonts. It appeared that sexual differentiation was genotypically determined and there were male and female schizonts. As further evidence to support their findings, they stated that although they found micro- and macrogamonts sharing a single host cell, the gamonts occupied separate parasitophorous vacuoles and thus they must have originated from different schizonts. Merozoites themselves showed no structural differences that would indicate their ultimate sexual roles. As the gamonts matured they became gametocytes and eventually gametes. One macrogamont developed into a single macrogamete, but one microgamont gave rise to many microgametes. Colley (1967) studied the microgametogenesis of Eimeria nieschulzi in rats. He described the development of the microgamont in the parasitophorous vacuole. According to Colley, the microgamete underwent a series of nuclear divisions, with
the newly formed nuclei moving to the periphery of the gamont. He also reported the location of three basal bodies between the cell membrane of the gamont and the peripheral nuclei, and during later development, these basal bodies gave rise to the flagella. In addition, he described the protrusion of the nuclei from the gamont surface, with subsequent budding of free microgametes into the parasitophorous vacuole. Speer (1979) reported similar observations while studying gametogenesis in *E. magna* in rabbits and in cell culture. He further reported that microgametes became highly motile when exposed to bile salts, thus aiding in their escape from the parasitophorous vacuole. Once free from the host cell, the microgametes swam about, actively seeking out a macrogamete, to which they attached. At this point, fertilization occurred. The resultant zygote completed formation of an oocyst wall and the young oocyst then passed from the host with the feces, ready to sporulate and begin the cycle anew.

**Fertilization**

Austin (1975), in a lengthy review of the topic, defined fertilization in vertebrates, invertebrates, and protozoans as involving the union of male and female gametes by fusion of the limiting membranes of the two cells. The result is a new cell that is bounded by a membrane that is a composite, or mosaic, containing characteristics of both parent cell membranes. The author further stated that in mammals,
gamete fusion is preceded by the penetration of the protective outer investments of the egg by the spermatozoon. This is accomplished by the release of lytic enzymes carried in the acrosome, predominantly hyaluronidase, and also acrosin, catalase, carbonic anhydrase, lactic dehydrogenase, aryl phosphatase, aryl sulfatase, B-N-acetyloglucosaminidase and phospholipase A. He further stated that hyaluronidase is apparently the critical component for effecting penetration. Prior to actual penetration, attachment of the two gametes must occur. The first phase involves recognition and attachment of the sperm to the zona pellucida. This phase, referred to as B1, is rapid (within two minutes of mixing) and temporary. After about 30 minutes, a second phase, B2, occurs, and produces a more permanent binding. This is followed shortly by penetration of the sperm into the egg.

The fertilization events that occur in mammals, as described above, are reflected in other phyla as well. Colwin and Colwin (1961) reported that among invertebrates, specifically annelids, fertilization involves the recognition, attachment, and fusion of spermatozoa and egg membranes that results in the formation of a mosaic membrane that bounds the zygote. The initial contact between the gametes occurs when the acrosomal tubules of the spermatozoon indent the egg plasma membrane leading to interdigitation of the membranes and subsequent fusion. Hiwatashi (1967) described fertilization in the ciliated
protozoon *Paramecium* as a very precise and orderly
process that proceeds in a manner comparable to
fertilization in other organisms. It involves initial
adhesion, the control of fertilization specificity
(recognition), and initiates activation in the reproducing
animals. The major difference in *Paramecium* is that
fusion is between whole animals, not discrete gametes, and
union is temporary (conjugation).

The unique cell surface characteristics of gametes that
mediate recognition, adhesion, and penetration are a
recurring theme in the literature. Metz (1967) stated that
both sperm and egg surfaces are complex, and that the sperm
plasma membrane is a composite of substances or molecular
patterns (antigens) some of which have limited regional
distribution. He stated that the fertilizin-antifertilizin
theory dominated thinking at that time, despite the fact
that proof of the theory had been demonstrated in some, but
not all, species belonging to several phyla. It appeared
that fertilizin-antifertilizin (a specific antigen-antibody
system), which was thought to function in recognition and
gamete attachment, was widely, if not universally, accepted.
Colwin and Colwin (1967) believed that the initial contact
resulting in adhesion between a sperm and an ova was of the
molecular binding type, and accordingly, they considered it
unlikely that all the events of fertilization from
recognition to adhesion to zygote formation would be
mediated by one set of substances, specifically
fertilizin-antifertilizin, as proposed. They felt it more likely that several specific molecular interactions would be involved.

Recently, investigators began to scrutinize the complexities of the sperm surface coat. Tzartos (1979) reported that anti-sperm antibodies added to in vitro fertilization systems exhibited multiple inhibitory affects on sperm passage through egg investments, with no general harmful affects on sperm themselves. He speculated that the antibodies, therefore, must have been acting on sperm enzymes and/or receptors for egg recognition. The fact that the antibodies not only stopped the passage through the egg but released sperm into the culture medium favored the interpretation that the antibodies were directed against receptor sites rather than enzymes.

Tung et al (1980) stated that relatively little attention had been directed to the role of sperm surface molecules in the induction of the acrosome reaction (wherein lytic enzymes are released by the sperm). These investigators found that anti-sperm antibodies completely inhibited the acrosome reaction, and from these results, postulated that there were specific surface receptors for external ligands, including calcium, and that interactions between these receptors and other substances were prerequisites for the acrosome reaction. Yanagimachi et al (1981) offered more support for the postulate that many prefertilization and fertilization events involve antigenic
molecules on sperm surfaces, and the function of the receptor or ligand molecules may be interfered with by specific antibodies. They proposed several mechanisms of action. The antibodies may directly react with the receptor molecules and block binding of required substances. Alternatively, the antibodies may react with surface antigens that are not themselves involved in sperm-ovum interactions. By forming antigen-antibody complexes on the sperm surface membrane the receptors may become masked and inaccessible to surface molecules or other substances of the zona pellucida or vitelline membrane of the ovum. Examining another possibility, Phillips (1984) proposed a mechanism of action for sperm-egg attachment whereby acrosomal enzymes from the sperm hydrolyze substrates that would uncover receptor sites on the egg. Antibodies that inhibit the acrosome reaction would then prevent enzymatic unmasking of these sites and block attachment of sperm to the egg.

**Fertilization in the Sporozoaa**

Chobotar and Sholtyseck (1982), state that fertilization in the sporozoa is an anisogamous process. The event is rapid and thus frequent observation is difficult. According to these authors, the essential requirement is that the male microgamete contacts the female macrogamete and effects entrance thereto, although the details vary in different groups of sporozoans. Desser (1972) reported the entrance of the entire microgamete into the macrogamete in
Parahaemoproteus. Gallucci (1974) reported that in Haemoproteus columbae the plasmalemmas of the two gametes fused at the time of penetration, with the resulting zygote membrane being a combination of both micro- and macrogamete membranes. Sinden et al (1976) described fertilization in Plasmodium yoelii nigeriensis as being a three phase process. First was contact between the amorphous surface coats of the two gametes, followed by the second phase of plasmalemma fusion. Third was the entrance of the microgamete nucleus and axoneme into the macrogamete. Aikawa et al (1984) confirmed the fusion of the plasmalemmas of the two gametes of Plasmodium gallinaceum, with subsequent entrance of the microgamete nucleus into the macrogamete. They further described the microgamete nucleus as being surrounded by a channel of rough endoplasmic reticulum up to the point of nuclear fusion.

Fertilization in the Eimeriina has been described by several workers. Scholtyseck and Hammond (1970) reported that the entire microgamete of Eimeria bovis entered the macrogamete with a penetrating action. Fisher (1980) reported the microgametes of Eimeria tenella positioned themselves parallel to the surface of the macrogamete prior to incorporation, and after entrance, the microgametes were surrounded by a granular membrane-like complex resembling rough endoplasmic reticulum. Sheffield and Fayer (1980) described the fertilization of Sarcocystis bovicanis wherein the fertilizing microgametes were found between the
two outer layers of the macrogamete (thought to be of host origin) and the inner layer. The latter appeared to be discontinuous at the point of contact between the two gametes. The microgamete was surrounded by a single membrane plasmalemma and was oriented tangentially to the macrogamete. The two gametes were actually connected by a small neck-like region through which there was cytoplasmic continuity. In the neck region the gamete membranes formed an electron-dense ring through which the microgamete nucleoplasm passed into the macrogamete cytoplasm. They also stated that the earlier reports of Madden and Vetterling (1977) of penetration of the macrogamete by the microgamete may have been the position of the microgamete between the two outer host layers and the inner parasite layer of the macrogamete surface. Chobotar and Sholtyscek (1982) also reported membrane fusion and neck-ring formation in Sarcocystis species. The early stages of fertilization in Eimeria maxima are described by Elwasila (1983). He reported the microgametes as approaching the macrogamete with their nuclei directed forward. At the site of contact between the two gametes, the membranes of both appeared to change and dissolve to facilitate the entry of the microgamete. Paterson and Desser (1984) distinguished two patterns of fertilization in the suborder Eimeriina. In Eimeria iroquoina and Eimeria bovis the microgametes appeared to be internalized intact within the macrogamete before fusion of
the gamete plasmalemmas occurred. In *Eimeria maxima* and *Sarcocystis* species, it appeared that the gamete plasmalemmas fused prior to internalization of the microgametes. In their study they found the microgametes to be surrounded by membrane structures resembling rough endoplasmic reticulum.

Hybridoma Technology

Since the introduction of monoclonal antibodies in 1975, the literature has swelled with examples of their production and use. For a concise encapsulation of the theoretical principles of monoclonal antibody production, the reader is referred to Goding (1982). The practical application of cell fusion occurred in 1975 when Kohler and Milstein demonstrated that fusion between normal antibody secreting cells and myeloma cells could be achieved using Sendai virus. The resulting cells continued to secrete immunoglobulins, as in the spleen cells, and retained the malignant properties of the parent myelomas. From their studies they concluded that any antibody secreting cell could be cloned and immortalized if that cell could be isolated and expanded. They then described the hypoxanthine, aminopterin, and thymidine (HAT) selection process. Spleen cells will die in culture after about two weeks. Myelomas will continue to thrive indefinitely and must be selected against. The main biosynthetic pathways for purines and pyrimidines can be blocked by the folic acid
antagonist aminopterin. The cell can still synthesize DNA via the "salvage" pathways. These pathways depend on the enzymes thymidine kinase (TK) and hypoxanthine phosphoribosyl transferase (HPRT). If the cell is provided hypoxanthine and thymidine, in the presence of the enzymes, DNA synthesis can occur. If one or the other enzyme is lacking, DNA synthesis ceases. The cell can be rescued by fusion with another cell that supplies the missing enzyme. Thus, if spleen cells (which possess TK and HPRT, but die in culture) are fused with myeloma cells lacking TK and HPRT, only the hybrid cells will survive in HAT medium.

The techniques involved in production and growth of hybridomas are quite standardized. The major difficulties with the procedure arise with the selection of an appropriate target antigen. Warr (1982) stated that antibodies, and the cells that secrete them, recognize an antigen primarily by the conformational determinants on the surface of the molecule. He also estimated that an animal's immune system could, theoretically, recognize and assemble corresponding antibody molecules to perhaps 10 antigenic determinants. In further consideration of the antigen selection problem, Greaves (1984) stated that it is important to appreciate that different regions or subunits of antigens are not equipotent immunologically; as far as a mouse immune response is concerned, certain areas of an antigen will be immunodominant. It is therefore desirable, though not necessary, to use a pure and highly characterized
antigenic preparation to immunize mice for hybridoma production. This would increase the probability of the mouse producing the desired antibody, and would ease the screening process once antibody was obtained.

**Application of Monoclonal Antibody Technology in Parasitology**

Since Kohler and Milstein (1975) described the procedure for producing monoclonal antibodies, these substances have found broad acceptance and use in many areas of immunologic research. In the field of parasitology they have been used extensively. Cain (1984) reported that heavy emphasis has been placed on the development of vaccines to several animal and human diseases caused by animal parasites. Monoclonal antibodies have been widely used to help isolate parasite immunogenic antigens that are used to produce the vaccines. He also described the following strategies that have often been employed: 1) monoclonals have been made against parasites and then correlated with sera from functionally immune hosts; 2) monoclonals have been screened in cytotoxicity assays using eosinophils or other anti-parasite components of the immune system; 3) monoclonals have been shown to confer protection on passive transfer to the appropriate host; 4) it has been possible in some instances to neutralize infection by prior incubation of the parasite in vitro with monoclonals; 5) monoclonals have been used in affinity chromatography to isolate and purify immunogenic
antigens. The author also cataloged the areas of ongoing research in parasitology where monoclonal antibodies have been widely used, specifically, malaria, trypanosomiasis, leishmaniasis, giardiasis, schistosomiasis, fascioliasis, trichinelliasis, ascariasis, and filariasis.

In 1976, Gwadz demonstrated successful immunization against the sexual stages of malaria by immunizing chickens with infected red blood cells that had been treated with formalin or X-rays. In 1978, Green did further work with simian malaria and showed that Rhesus monkeys produced blocking antibodies effective against both sexual and asexual stages of the parasite. Carter et al (1979) purified the antigens necessary for inducement of antimalarial antibodies in chickens using *Plasmodium gallinaceum*, and this work led directly to the production, by Rener et al. (1980) of monoclonal antibodies to these specific malarial antigens. These monoclonal antibodies were highly effective in blocking fertilization of the parasite harbored in the mosquito gut. Yoshida et al (1980), and Yoshida et al (1981), using monoclonal antibodies developed against *Plasmodium berghei*, were able to characterize the protective antigens on the surface membrane of the sporozoites. Aikawa et al (1981) used monoclonal antibodies directed against the gametes of *Plasmodium gallinaceum* to characterize and isolate their surface antigens. Danforth et al (1982) produced monoclonal antibodies of both IgG and IgM isotypes to the surface
antigens of *Plasmodium berghei* sporozoites. This was preliminary work in the production of an irradiated vaccine model. Kasper et al (1984) used monoclonal antibodies to identify and characterize stage-specific surface antigens on *Toxoplasma gondii* sporozoites. Pearson et al (1984) have used monoclonal antibodies to study the antigenic sites on the major variable surface glycoproteins of bloodstream and metacyclic forms of African trypanosomes. These studies had the objectives of understanding the antigenic polymorphism and the extent of the antigenic repertoire of the metacyclics. The results of these studies suggest new possibilities for vaccination strategies despite the supposed impasse established by the phenomenon of antigenic variation.

In the area of *Eimeria* research, Danforth (1982) produced monoclonal antibodies to surface and internal antigens on the sporozoites and merozoites of *Eimeria tenella* and *Eimeria mitis*. Speer et al (1983) produced monoclonal antibodies to surface antigens of sporozoites, merozoites, and oocysts of *Eimeria tenella*. The possible production of an anti-coccidial vaccine in the near future is a direct result of Danforth's work.
Immunoelectron Microscopy
Techniques Involving Colloidal
Gold Labeling

Robinson et al (1984) reported that gold spheres ranging in size from 15 to 150 nm in diameter have been used increasingly as markers for indicating the location of macromolecules in specimens examined by light, transmission, and scanning electron microscopy. Gold particles have been used in either direct or indirect labeling procedures to detect the presence and location of antigens. Bauer et al (1975) stated that protein molecules were adsorbed around colloidal gold particles in a sterically favorable position necessary for antigen-antibody reactions, and gold particles were readily visible in the TEM even without negative staining. Horisberger et al (1975) described the drawbacks of other cell surface labeling techniques used in EM, specifically, peroxidase, latex beads, haemocyanin molecules, and ferritin. These techniques were found to be either laborious to prepare or not suited to both modes of EM. However, the authors stated that, on the other hand, the technique of conjugating protein or antibody to colloidal gold particles has proven simple and effective. The main advantage of a heavy metal label is its strong emission of secondary electrons, thus rendering it easily distinguishable from cell surface structures, which are low emitters of secondary electrons. Additionally, when used in SEM preparations, good spatial relationships were attained
by using the colloidal gold technique. The advantages of gold markers over ferritin were enumerated by Horisberger and Rosset (1977). They report that only a small amount of macromolecules (protein or antibody) were necessary to prepare the markers, and that non-specific adsorption of gold conjugates is very low when compared to ferritin. The authors stated that successful marking experiments are quickly visualized without a microscope since the labeled specimens are red to black, depending on the density and size of the marker particles. The fact that gold particles can be produced in several diameters makes it possible to do double-labeling experiments on a single specimen. The value of variable size gold particles to immunocytochemistry is further described by Slot and Geuze (1981). These investigators found that large particles were easily observed at low magnifications, so that a good overall impression of the labeling pattern could be obtained. The large particles were also more distinct over electron dense structures than small ones. Small particles were found to have the advantage of higher sensitivity in display of the immune reaction, and less likelihood of obscuring small receptor sites.

Recent works in immunoparasitology that incorporate the colloidal gold technique include the study by Fine et al (1984) involving monoclonal antibodies that localized protective antigens and their precursors on the sporozoites of Plasmodium knowlesi, the study by Oka et al (1984)
that used monoclonal antibodies to localize protective antigens on the merozoites of *Plasmodium yoelii*, and the work of Speer et al (1983) with *Eimeria tenella* cited above.
EXPERIMENTS

Experiment I:
Production of Hybridomas

Concept. Production of a hybridoma secreting a monoclonal antibody specific for microgametocytes of *Eimeria tenella* was the primary objective of this project. The initial task was to develop an antigen preparation that would be suitable for immunization of the mice, and for antibody assays once the hybridomas were available.

Materials and Methods. The antigen used to hyperimmunize the mice was obtained by growing *Eimeria tenella* in cultured chick kidney cells using the procedure of Doran (1970) until the fourth and fifth day post infection. At that time, the cell culture supernatant was harvested and examined for the presence of microgametocytes. Supernatants were pooled and stored at -80°C until sufficient microgametocytes were on hand to immunize two mice using approximately $8 \times 10^4$ microgametocytes per mouse per immunization.

Two BALB/c mice, one male and one female, each approximately eight weeks of age, received three immunizations with the antigen preparations described above by the intraperitoneal route at two week intervals. For the third immunization, the antigen was mixed 1:1 v/v with Freund's complete adjuvant. Three days prior to sacrifice, the mice received a booster immunization of antigen (without
adjuvant) by tail vein injection. The mice were sacrificed by cervical dislocation and the hyperimmunized spleens were removed and transferred to sterile DMEM (Dulbecco's Minimal Essential Medium; Gibco Labs.). The spleens were disrupted by scraping and forcing them through a 25 gauge needle using a 12 ml syringe until a cell suspension was obtained. The suspension was washed twice in DMEM, counted, and set aside. At this time, SP2/0 mouse myeloma cells (courtesy of Dr. Mark C. Healey, Departments of Animal, Dairy, and Veterinary Science, and Biology, Utah State University) were washed and counted, and mixed with the spleen cells at a ratio of approximately 10 spleen cells to one myeloma cell. This mixture was washed once in DMEM and the cell pellet gently resuspended in 1 ml of 30% polyethylene glycol in DMEM and immediately centrifuged at 500 g for three minutes. The pellet was resuspended in DMEM, which was added drop-wise to avoid osmotic shock to the fused cells. The pellet was washed in DMEM and resuspended in HAT (hypoxanthine, aminopterin, thymidine) medium, and the cells plated into 96 well microtiter plates (Falcon®) at a concentration of $3.3 \times 10^5$ cells per well. The plates were incubated at 37°C in 10% CO$_2$. The cells were fed periodically with HAT medium and after two weeks of incubation the wells were examined for colony growth. Of four plates prepared, 40 wells showed positive growth. The colonies from these wells were expanded to 24 well plates (Falcon®) and transitioned through HT medium to DMEM for maintenance.
Those expanded wells showing positive growth were assayed for specific antibody production. Positive wells were subcloned by limiting dilution, re-assayed, and expanded to flasks for maintenance.

Results. Of the four 96 well plates originally prepared, 40 wells showed positive hybridoma colony growth after two weeks of incubation in HAT medium. These wells were expanded to 24 well plates and assayed for specific antibody production by IFA (indirect immunofluorescent antibody assay). Many of these hybrids produced antibodies to chick kidney cell material, merozoites, and undefinable target antigens along with antibodies to the microgametocytes. Those showing positive results to the microgametocytes were subcloned and re-assayed by IFA. The resultant subcloning yielded four positive clones, T1A3B9, T1G6G12, T2B5E1, and T2B4D11. Of these four, T1A3B9 gave by far the strongest IFA reaction (see Figures 1-5). This clone was expanded to 250 ml flasks for maintenance and production of supernatant. When sufficient cells were available in maintenance cultures, samples were frozen at -80°C for long term storage, and others were inoculated into pristane (2,6,10,14-tetramethyl pentadecane) primed mice for production of ascites fluid.
Experiment II: Determination of Specificity of Antibody

Concept. Once the hybridoma colonies were growing successfully, the cell culture supernatants from the positive wells would have to be assayed for presence of antibody and also for antibody specificity. Because a mixed antigen was used to immunize the mice, an ELISA (enzyme-linked immunosorbent assay) was ruled out as an initial screen. The alternative method selected was an IFA, wherein the target cells could be visualized under fluorescent microscopy for positive identification. A concurrent test for antibody characterization, the IEP (immunoelectrophoresis) assay was also done.

Materials and Methods. Ceca from White Leghorn chicks infected with *Eimeria tenella* were, on days four and five post-infection, surgically removed, placed in a wax dissecting pan, and longitudinally opened to expose the mucosal surfaces. The mucosa was gently scraped with a spatula so as to remove only the most superficial layer of tissue. These scrapings were washed in DPBS (Dulbecco's phosphate buffered saline, Gibco Labs) and applied as a smear to glass microscope slides. The smears were allowed to air dry. The slides were washed in DPBS, and the test supernatant applied and incubated at 37°C for 45 minutes. The slides were again washed in DPBS, flooded with a goat anti-mouse IgG conjugated with FITC (Litton-Bionetics), and incubated at 37°C for 45
minutes. The slides were then washed three times in fresh changes of DPBS, blotted dry, cover slips mounted with IFA mounting medium (90% glycerol in 10% DPBS, pH adjusted to 8.6), and observed on a Zeiss® inverted microscope equipped with epifluorescence illumination.

Another series of IFA were run using frozen sections of *Eimeria tenella* infected chick ceca. Frozen sections of 6-8 um thickness were cut on an IEC cryostat, mounted on glass microscope slides, and processed as described above.

Supernatant from wells showing positive growth was subjected to immunoelectrophoresis (IEP) to determine if antibody was present, and if so, the isotype and subisotype of the antibody. The electrophoresis was performed by placing a clean glass plate pre-coated with .01% agarose on a level surface and gently warming the plate. A solution of 200 mg of agarose A in 20 ml Tris-barbiturate buffer (pH 8.6) was poured onto the warm plate and allowed to solidify. When the gel was solid, five circular wells were cut, and between these wells six troughs were cut. Approximately 25 μl of test supernatant were placed into the wells. The plate was then placed into the electrophoresis chamber, the electrodes connected, and the plate electrophoresed at 100 volts for two hours. At the end of the electrophoresis period, the plate was removed from the chamber and antisera to mouse IgG, IgM, IgG1, IgG2a, IgG2b, and IgG3 were added, respectively, to the troughs. The plate was placed into a humidified chamber at room
temperature for 24 hours and observed for precipitin arcs. The plate was then washed in PBS, air dried, and stained with Amido black.

Results. The IFA's showed a strong positive reaction on the microgametocytes taken from cecal scrapings and those in situ in the frozen sections of ceca (Figures 1 and 3). Negative controls showed only background illumination, (Figures 2 and 5), although oocysts in all preparations exhibited a slight fluorescence believed to be refractory in nature. No other life cycle stages of the parasite showed any reaction with the monoclonal antibody.

Results of the IEP of the T1A3B9 hybridoma supernatant showed the antibody to be IgG of subisotype IgG2b (Figure 9).

Experiment III: Evaluation of Immunologic Potency of the Antibody

Concept. Once the specificity of the antibody had been determined, it was necessary to test the immunologic activity. If the antibody was specific for the microgametocytes, it might exhibit some inhibitory effects on the ability of microgametes to complete their development and subsequently fertilize the macrogametes. It was decided to use in vitro oocyst production as an assay for immunologic activity.

Materials and Methods. White Leghorn chicks of approximately two weeks of age were sacrificed by cervical
dislocation. The kidneys were removed and placed in medium A which consisted of Hank's Balanced Salt Solution (Gibco), 80%; lactalbumin hydrolysate, tissue culture grade, (Gibco), 10% in HBSS; and 10% fetal bovine serum (HyClone Labs). The kidneys were diced with sterile razor blades until the pieces were about 1 mm³, and then transferred to a trypsinization flask containing 10 ml of trypsin-versene (TV) solution (0.2% versene plus 0.5% trypsin 1-300 powder in 0.9% physiological saline solution). The tissue was trypsinized with agitation for 15 minutes at 37°C. The supernatant was discarded and 10 ml of fresh TV solution was added. The tissue was trypsinized as above for an additional 15 minutes, the supernatant discarded, and replaced with 10 ml of fresh TV solution. The tissue was then trypsinized a third time for three minutes, the supernatant discarded, replaced with fresh TV solution, and trypsinized a final time for three minutes. Supernatant from the final trypsinization was added to 10 ml of medium A, centrifuged at 500g for five minutes, resuspended in 10 ml of medium A, and the suspended cells counted on a hemocytometer. The cell suspension was diluted to a concentration of 5.0 x 10⁵ kidney cells per ml of medium A. One ml of this suspension was seeded into sterile plastic Leighton tubes (Costar®) containing two ml each of medium A. The tubes were incubated at 41°C in 7% CO₂ for 24 hours. At this time, growth medium A was replaced with maintenance medium B which consisted of Hank's
Balanced Salt Solution (Gibco), 90%; lactalbumin hydrolysate, tissue culture grade, (Gibco), 5%; and fetal bovine serum (Hyclone Labs), 5%. The decreased richness of the maintenance media was designed to prevent overgrowth of the kidney cells before the parasites completed their life cycle. Both media systems contained potassium penicillin G (100 i.u./ml), streptomycin sulfate (100 ug/ml), and phenol red indicator (100 ug/ml).

When the primary kidney cell cultures had reached approximately 25%-50% confluence, they were inoculated with sporozoites of *Eimeria tenella* (Beltsville Strain #25) by the following technique. Oocysts of *Eimeria tenella* were cleaned in sodium hypochlorite for 25 minutes, washed several times in Puck's saline A, and disrupted mechanically in a tissue grinder. Free sporozoites were placed in excystation medium (0.025g trypsin plus 0.075g sodium glycotaurocholate in 10 ml Puck's saline A) at 41°C and checked every 15 minutes for presence of sporozoites. When sufficient sporozoites had excysted, the excystation medium was passed through a nylon wool (Fenwal Labs) column to remove debris and purify the sporozoite suspension (Larsen et al., 1984). The filtrate was centrifuged at 500g for eight minutes, the pellet was resuspended in medium B, and the sporozoites were counted on a hemocytometer. Approximately 1.0 x 10^5 sporozoites were inoculated by pipette into each Leighton tube. After four hours, the medium was replaced with fresh medium B and subsequently
changed every 24 hours throughout the experiment. On days four, five, and six post-infection, the experimental tubes received one ml of hybridoma supernatant containing the test antibody along with the normal media change. Half of the control tubes received one ml of SP2/0 supernatant and the other half received no additives at all with the normal media changes. On day seven or eight post-infection, all plastic cover slips were removed from the tubes, fixed in 95% methanol for 15 minutes, washed in water, and stained with Wright-Geimsa stain for 30-45 minutes. The cover slips were allowed to air dry, and then mounted cell side down with Duco cement on glass microscope slides. Oocyst counts were then made on a light microscope.

Results. For day four, five and six experiments, a total of 40 in vitro trials were conducted using 20 experimental tubes and 20 control tubes. The mean oocyst production per tube for the controls was 30, while the mean for the experimental (antibody treated) tubes was 11, showing a greater than 50% reduction in oocyst production in the treated tubes. When the data were subjected to a Mann-Whitney U-Test, the results were statistically significant (P < 0.01; Figure 10).
Experiment IV: Determination of Antibody Binding Site Specificity

Concept. Having confirmed immunologic activity for the antibody, it was then necessary to determine the mechanism of action of the antibody. This would require localization of the binding sites of the antibody on the target microgametocytes by immunoelectron microscopy. Additionally, any pathological changes that occurred in the ultrastructural morphology of the target cells would be observed. The IEM study would also offer additional confirmation as to whether binding of the antibody occurred on life cycle stages other than the microgametocyte.

Materials and Methods. The IEM techniques involving colloidal gold labeling used in this study have been adapted from Fine et al (1984). Tissue samples for transmission electron microscopy (TEM) were obtained from various sources. Cultures of primary chick kidney cells, infected with *Eimeria tenella*, were treated with monoclonal antibody on days four, five, and six post-infection. On the sixth day, after the antibody treatment, the cells were gently trypsinized (or scraped with a cell scraper) to loosen them from the cover slips and to prepare a cell suspension. The cells were then washed once in TRIS/BSA (hydroxymethyl aminomethane/bovine serum albumin) buffer, pH 8.6, and resuspended in a 1:5 dilution of TRIS/BSA buffer and GAM 40 goat anti-mouse IgG conjugated with colloidal gold, 40nm particle diameter (obtained from Janssen Life
Sciences Products, through SPI, Inc.). The cells were incubated in the gold conjugate for 45 minutes at 41°C. In an alternative method, infected cells were suspended in growth medium, incubated with monoclonal antibody for 45 minutes at 41°C, then washed in TRIS/BSA buffer, resuspended in gold conjugate, and incubated again for 45 minutes at 41°C. Other samples were collected during *Eimeria tenella* excystation procedures. These samples consisted of relatively pure suspensions of sporozoites that were used as negative controls. These samples were processed by the methods already described. Still other samples were collected by scraping the epithelial linings of *Eimeria tenella* infected chick ceca with a spatula. These scrapings were then processed and labeled as described above.

After labeling, the tissue samples were prepared for TEM in the routine manner (Youssef, 1976). Samples not already fixed were fixed in 2.0% glutaraldehyde in TRIS/BSA buffer for one to two hours depending on sample size. They were then washed three times in buffer and post-fixed in a 1.0% solution of osmium tetroxide in TRIS/BSA buffer for one hour. The samples were washed twice again in buffer and then dehydrated through a graded series of ethanol, followed by soakings in propylene glycol, and then embedded in Epon/Araldite epoxy resin. Gold to silver sections were obtained using a glass knife in a Porter-Blum MT-2 ultramicrotome. Sections were mounted on copper grids and
then stained with uranyl acetate and lead citrate. The thin sections were then observed on a Zeiss EM9A electron microscope.

Tissue samples for scanning electron microscopy (SEM) were obtained as frozen sections, approximately 10 um thick, of *Eimeria tenella*-infected chicken ceca taken on days four, five, and six post-infection. The frozen sections were mounted on tissue culture grade plastic cover slips and immediately fixed in 2.0% glutaraldehyde in TRIS/BSA buffer for 30 minutes to one hour. Samples were washed twice in TRIS/BSA buffer, then incubated with monoclonal antibody for 45 minutes at 41°C. The samples were again washed in buffer, then incubated with a 1:5 dilution of GAM 40 in TRIS/BSA buffer for 45 minutes at 41°C. After two washings in buffer, the samples were dehydrated through a graded series of ethanol followed by transition through a graded series of ethanol/Freon® 113 mixes. After a soaking in pure Freon® 113, the samples were dried in a Polaron® critical point drier using Freon® 13 as a drying medium. Dried samples were mounted on aluminum stubs, and sputter-coated with gold in a Polaron® E5000 SEM Coating Unit. Prepared samples were observed in an AMR 1000 scanning electron microscope.

Results. Material consisting of antibody-treated sporozoites showed no indication of an antigen-antibody reaction. The sporozoite membranes were not labeled with gold particles. Although random gold particles did appear in
the preparation, their positions were not consistent with a labeling reaction (Figure 6).

Pellets of infected cecal scrapings that were treated with monoclonal antibody showed a distinct gold labeling reaction along the outer membrane of the microgametocytes, (Figures 7 and 8), and a negative reaction on all other tissues and parasite life cycle stages including macrogametocytes. The same results were obtained with infected primary chick kidney cell culture material. In all positive samples, the gold marker appeared to be localized on the membranes associated with the developing microgamete flagella. In previous studies using monoclonal antibodies specific for asexual stages (Danforth, 1982; Speer et al., 1983), the binding sites were distributed almost evenly over the surface of the target cells, and in some instances, internally (Danforth, 1982). In this current investigation, all markers were arranged in small clumps that appeared in spotty fashion over the flagellar membranes and in close approximation to the microgametocyte limiting membrane (Figures 7 and 8).

Samples of day four post-infection material examined by SEM showed early parasite life cycle stages (merozoites and very early immature gamonts) totally lacking any gold label, as was the host tissue (Figure 12). Day five material showed mature microgametocytes that were distinctly gold labeled, although other life cycle stages and host tissue were devoid of gold (Figures 11 and 13). Day six material
showed results identical with day five samples. As in the TEM results, gold marker was distributed in patches or clumps that appeared on or near the flagella of the developing microgametes, and on the microgametocyte limiting membrane (Figures 11 and 13).
DISCUSSION

Production of Hybridomas

The successful production of hybridomas secreting monoclonal antibodies to antigens derived from *Eimeria tenella*, as seen in this study, is consistent with results obtained by other investigators (Danforth and Augustine, 1981; Danforth, 1982; Speer et al, 1983), with the significant difference being that this current work is the first report of the sexual stages of an *Eimerian* parasite being used as the target antigen. Previous examples of production of monoclonal antibodies specific for microgametes of protozoan parasites were limited to work with *Plasmodium* species (Aikawa et al, 1981; Rener et al, 1980).

The selection of the microgametocyte stage of *Eimeria tenella* as the target antigen presented the problem of antigen purification. It is desirable, though not necessary, to use as pure a preparation of immunizing antigen as possible when preparing hybridomas (Warr, 1982; Greaves, 1984). The extremely laborious procedures needed to prepare a pure isolate of a single parasite life cycle stage (James, 1980), with the exception of oocysts and sporozoites, led to the decision to use a mixed antigen as the initial inoculum.

Two sources of inoculum were considered; first were
cecal scrapings from infected chickens, and second were parasite-containing supernatant from infected cell culture systems. The cecal scrapings provided large numbers of microgametocytes, and were relatively easy to collect and maintain. The major disadvantage was the extreme amount of contamination by bacteria, other microorganisms, host tissue debris, and food residue. Inoculum from in vitro systems was more difficult to produce and maintain, but provided a much cleaner preparation for immunization, and was therefore selected.

The supernatant from the technically more difficult in vitro system that was used as the inoculum contained host material in the form of chick kidney cells, some sporozoites that had not penetrated host cells and had subsequently failed to develop, schizonts primarily of the second and third generations, merozoites belonging to the second and third generations, free microgametes, and both micro- and macrogametocytes. Various macromolecular constituents of the growth media and fetal bovine serum were also present. All these components were assumed to be immunogenic and therefore capable of stimulating an antibody-producing response in the mice, as evidenced by the previously cited works of Danforth and Speer. It was believed, however, that the microgametocytes would be at least as immunogenic as the other components, based on reports of other investigators regarding the antigenicity of sperm (Metz, 1967; Jones, 1980; Haas et al, 1981) and the microgametes of
Plasmodium species (Rener et al., 1980; Aikawa et al., 1981).

The results obtained from the mouse immunization regimen confirmed the initial supposition regarding the immunogenicity of microgametocytes. Out of four 96-well plates inoculated, 40 successfully growing hybridomas were produced. Four of the hybrids were producing monoclonal antibodies of high specificity for microgametocytes. The IFA used as an initial screen for antibody specificity showed the strongest positive reaction on the areas of the microgametocyte where developing microgametes were budding forth from the limiting membrane (Figure 3). The IFA results were further supported by IEM assays using colloidal gold. Here, the marker was detected on the microgametocyte limiting membrane in close association with microgamete flagella (Figures 7, 8, 11, and 13). This finding is consistent with reports of other investigators who found that, in mammalian sperm, the tailpiece (flagellum) is a site of strong antigenicity (Haas et al., 1981; Jones, 1980). The possibility that the IEM results could have been artifactual was considered and dismissed. The validity of the colloidal gold immunolabeling technique is well documented (Horisberger et al., 1975; Speer et al., 1983; Fine et al., 1984; Oka et al., 1984), thereby reinforcing the credibility of the results obtained in this study.

In order to determine the isotype and subisotype of the monoclonal antibody, an immunoelectrophoretic assay was
conducted. Results of the IEP revealed the antibody to be an IgG2b. This is not consistent with the type of immune response to *Eimeria tenella* infections in chickens. Rose et al (1984) described a phased array of immune responses to infections of *Eimeria tenella*. The early phase, that prior to sporozoite penetration, probably involved secretory IgA produced in the intestinal and cecal mucosa. The second phase involved migration of sporozoites from the surface epithelium to the crypts provided no direct evidence of immune involvement, although the effects of IgA were probably still felt. The third phase involved immune responses to schizogony. That stage was probably the target of both secretory IgA and humoral IgG and IgM antibodies. There was also evidence that a dialysable transfer factor might have been active in the immune response. Davis et al (1978) proposed a larger, more critical role for secretory IgA in the immune response to *Eimeria tenella*, and a back-up role for humoral antibodies that might prevent the infection from spreading rampanty. Straneva (1984) described the major role of macrophages in immunity to *Eimeria tenella*. In light of these studies, it seems unlikely that, in a naturally occurring *Eimeria tenella* infection, an IgG class antibody would come into contact with the sexual stages of the parasite.
Stage and Site-specificity of the Monoclonal Antibody

The results of the IFA and IEM assays conducted on the monoclonal antibody produced in this study demonstrate a distinct stage-specificity. This differs with the reports of other workers (Danforth and Augustine, 1981; Danforth, 1982; Speer et al, 1983) who, using monoclonal antibodies to Eimeria tenella and other eimerian species, found antigenic determinants that were common to several life cycle stages, specifically, merozoites, sporozoites, sporocysts, and oocysts. In contrast, there are reports of stage-specific antigens occurring in Toxoplasma gondii and Plasmodium species (Aikawa et al, 1981; Rener et al, 1980; Kasper et al, 1984). It is noteworthy that the stage-specific antigens found in Plasmodium species were associated with the gamete stage of the life cycle, whereas the common antigens found in the eimerians were all associated with asexual stages.

Several explanations for a stage-specific antigen on the microgametocytes of Eimeria tenella can be formulated. The mature microgametocyte and free microgamete are the only stages in the life cycle that possess flagella. As the flagella protrude from the surface of the microgametocyte, they may offer unique conformational sites of sufficient antigenicity to elicit an antibody response. These sites may be intrinsic to the flagellar membranes, they may be
associated with the basal structures of the flagellum, or they may be associated with the limiting membrane of the microgametocyte. In this case, the distortion of the limiting membrane during budding may expose normally obstructed sites of high antigenicity. The nature of the sites themselves is conjectural at this point, but they may be integral membrane proteins or glycoproteins that serve as ligand sites for ionic or macromolecular substances of either host or parasite origin that, in some way, assist in the budding and release of microgametes.

In his description of microgametogenesis, Scholtyseck (1979) reported that as the developing microgametes protrude from the microgametocyte, their anterior portions are oriented outward, and were therefore the first to bulge into the parasitophorous vacuole. It is at this anterior end of the microgamete that the perforatorium is located. The author described the perforatorium as an organelle that probably functions as an aid to the penetration of the macrogamete by the microgamete. As such, it may be considered as analogous to the acrosome in the sperm of higher organisms. As previously stated, the acrosome is a very antigenic site on these sperm, and therefore, so might the perforatorium of the protozoan microgamete be of considerable antigenicity. This interpretation would be consistent with the results of the IFA assays, which showed the small buds on the surface of the microgametocyte as having the strongest fluorescence.
Fisher (1980) stated that the flagella of the microgametes of *Eimeria tenella* were the first organelles to approach the macrogamete prior to fertilization. This may indicate the presence of receptor sites on the flagellar membrane that would act in cell-cell recognition and attachment. Singer (1976) and Gerisch (1977) stated that cell recognition and adhesion could be traced to specific loci on the surface membranes. These sites, which were very antigenic, probably represented integral membrane proteins or glycoproteins that acted as ligands for adhesion.

The results of the immunoelectron microscope study show a definite stage and site specificity for the target antigen recognized by the monoclonal antibody. Gold marker only appears on the microgametocyte stage, and only on the limiting membrane of the microgametocyte in close proximity to flagella and the anterior budding portions of the microgametes. This leads to the conclusion that the target antigen is 1) a receptor site functional in cell-cell recognition and/or adhesion between the microgamete and the macrogamete; 2) a site of enzyme production that may function in attachment and/or penetration of the macrogamete by the microgamete; 3) an integral structure of the limiting membrane of the microgametocyte that is exposed during a conformational change of the membrane during budding of the developing microgamete.
Proposed Mechanism of Action of Monoclonal Antibody in Blocking Fertilization

As discussed earlier, fertilization is a complex process involving the union of male and female gametes by fusion of the limiting membranes of the two cells. Prior to fusion, there must be recognition and attachment of the gametes. In order to effect this union, the cell surfaces of gametes are invested with unique, highly specific receptor sites, enzyme systems, and ligand, or binding, sites for extrinsic substances such as ions. Disruption of structures or function of any of these elements may result in failure of gamete union and a blockage, or inhibition, of fertilization (Tzartos, 1979; Tung et al, 1980; Phillips, 1984).

To determine if the antibody was exerting an effect on the ability of the microgametes to fertilize macrogametes, a series of in vitro trials was conducted. Results of these experiments showed a greater than 50% reduction in the number of oocysts produced in the antibody-treated tubes as compared to controls (Figure 10), thus indicating a definite inhibitory effect by the antibody on fertilization. A 50% reduction in oocyst production, rather than a greater percentage, is consistent with current immunologic thinking. The target of the monoclonal antibody, the microgametocyte, spends a considerable amount of time in a sequestered state inside the host cell, where it is not exposed to the
antibody. In addition, not all microgametocytes become exposed to the extracellular milieu at the same time. If antibody is not present in sufficient concentrations for the entire period of microgametocyte presence, then some of the targets may escape antibody attack and go on to produce viable, functional microgametes capable of completing fertilization. This situation is very likely the explanation for the results obtained in this oocyst assay experiment. Based on the results of antigen localization assays described earlier, three distinct potential mechanisms of action of the monoclonal antibody can be postulated, these being 1) integral membrane proteins; 2) membrane-associated enzyme systems; and 3) receptor sites.

If the target antigen is an integral membrane protein of the microgametocyte limiting membrane that is exposed by conformational distortions of the membrane during budding of the microgametes, then antibody directed against this IMP could act as an inhibitor. If cross-linking of several IMP sites is effected by the antibody, then the release of the microgamete from the gametocyte surface could be prevented by physical or electrostatic means. This type of action would lead to retention of mature microgametes on the gametocyte surface, or possibly, release of damaged microgametes into the cecal lumen or tissue culture medium. As yet, no such evidence has been found in IEM studies, and therefore this proposed mechanism of action is the least likely to actually exist.
If the target antigen is an IMP of the microgametocyte, and subsequently, the microgamete limiting membrane that acts as a ligand site for energy-rich substrates required for mitochondrial enzymes of the microgamete, then cross-linking of these IMP might adversely effect the permeability of the membrane, or destroy transmembrane transport systems, for these substances (Fawcett, 1977). Should this be the case, no overt pathological changes need be displayed, yet microgamete function could be severely curtailed, thus resulting in decreased incidences of fertilization.

The existence of enzyme systems on the sperm limiting membranes from organisms of several phyla is well documented. Austin (1975) described a battery of enzymes associated with sperm membranes, and implicated hyaluronidase as a prime candidate for effecting zona penetration.

The enzymes found on the sperm limiting membrane have two major functions; first, the penetration of the egg outer investments by the sperm, and second, the actual attachment of sperm to the egg prior to membrane fusion. Working with mammalian systems, Tung et al (1980) and Phillips (1984) describe the enzyme systems that effect penetration and attachment of sperm to the egg. They implicate the battery of enzymes associated with the acrosome reaction as being essential precursors to fertilization. Florman et al (1983) report that specific inhibitors for the enzyme trypsin will
prevent the binding of sperm to eggs in *in vitro* mouse systems.

Among invertebrates, sperm enzymes are also essential for initiating fertilization. Hoshi et al. (1983), using sperm of *Ciona intestinalis*, an ascidian, found that glycosidases, specifically α-L-fucosidase, are required enzymes for sperm-egg binding. When the enzyme was inhibited, binding did not occur. A thorough review of the literature revealed no reported evidence of membrane-associated enzyme systems operating in the coccidia, however, this area deserves further scrutiny.

If the target antigen in this study is a membrane-associated enzyme, then the monoclonal antibody may be inhibiting the activity of that enzyme. The action may be due to a direct blocking of the enzyme by binding at the active site, or, if cross-linking of the antibody is involved, the active site may be masked or obstructed by steric changes in the molecule.

The prerequisite to the union of male and female gametes is cell-cell recognition and attachment (Austin, 1975; Colwin and Colwin, 1961; Hiwatashi, 1967; Metz, 1967; Tzartos, 1979; Yanagimachi et al., 1981). These important functions are mediated by specialized surface molecules referred to as receptor sites. They are present and operative in gamete union throughout several phyla, from protozoans to mammals (Hiwatashi, 1967; Hoshi et al., 1983; Colwin and Colwin, 1961). In early works (Metz, 1967), the
receptor system was likened to an antigen-antibody reaction and the substances were referred to as fertilizin-antifertilizin. Later, it was believed that no one system could be common in all cases, and that there must be species and gamete-specific receptors throughout all taxa (Colwin and Colwin, 1967).

The nature of the receptors themselves has been investigated (Singer, 1976; Gerisch, 1977) and they are thought to be integral membrane proteins or glycoproteins. The receptors may function in several ways. They may be strictly recognition sites, as in the case of sperm-zona pellucida recognition, wherein site conformation plays an important role (Yanagimachi et al, 1981). The same factors may apply in the situation where receptors function as attachment and binding sites that hold sperm to egg. Yet another mechanism of action for receptors would be as ligand sites for external substances that trigger reactions in the male gamete. An example is offered by Tung et al (1980) who found that receptor sites on the sperm membrane bind Ca++. This binding is necessary to precipitate the acrosome reaction (lytic enzyme release) in the sperm that facilitates zona penetration.

Having established the presence, nature, and role of receptor sites on sperm surfaces, the investigators previously cited have demonstrated the importance of these receptors by blocking them with specific antibodies. In every case of receptor blockage, sperm-egg recognition,
attachment, and fertilization were inhibited or prevented entirely.

If the target antigen in this study is a receptor site on the microgamete surface, the mechanism of action of the antibody may involve one of several possibilities. Blockage of the receptor by direct binding of the antibody to the active site may inhibit recognition of the macrogamete by the microgamete. It may also impair or prevent gamete attachment. If the antigen-antibody reaction involves cross-linking, then all the receptor sites may not be occupied by the antibody, but masking of a large number of sites by antigen-antibody complexes may occur, thus rendering the system inoperable. The antibody may, through similar action, prevent binding of essential ligands, Ca\(^{++}\) for example, to the gamete surface. Furthermore, it is unlikely that receptor sites would be distributed evenly over the entire surface of the microgametocyte or microgamete. The sites would probably be confined to specific regions of the surface membrane, which would be consistent with the patchy distribution of antibody binding sites found in this current study (Figures 7, 8, 11, and 13).

In conclusion, given the similarities in the fertilization process between organisms throughout all taxa, the Sporozoa and Eimeriina included, it seems probable that the operations and components previously described apply to the fertilization events in *Eimeria tenella*. When the
results of the immunofluorescent antibody assays, immunoelectron microscopy, and \textit{in vitro} antibody trials obtained in this study are examined, it appears that the monoclonal antibody under scrutiny is acting on either an enzyme system or receptor site on the microgametocyte, and subsequently, the microgamete, surface membrane. This antibody is blocking the operation of the antigenic site by either direct binding or, through cross-linking, by the formation of obstructive antigen-antibody complexes. The ultimate result is the inhibition of fertilization. Further research should be pursued in the area of antibody and antigen characterization. Separation techniques should be applied so that relatively pure antibody may be isolated and used in dose dependency studies. Purified antigen preparations should be investigated to determine the protective nature of the antigen, and its potential for use as a vaccine component.
LITERATURE CITED


Utah State University Press, Logan, Utah. 1-120.