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IN VITRO EVALUATION OF ANTI-*EIMERIA TENELLA*, GAMONT-SPECIFIC, MONOCLONAL ANTIBODIES AND PARTIAL CHARACTERIZATION OF THEIR TARGET ANTIGENS

by

Eric Wilson

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Biology

Approved:

UTAH STATE UNIVERSITY
Logan, Utah

1995

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Eric Wilson

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ABSTRACT

In Vitro Evaluation of Anti-*Eimeria tenella*, Gamont-specific, Monoclonal Antibodies and Partial Characterization of their Target Antigens

by

Eric Wilson, Master of Science

Utah State University, 1995

Major Professor: Dr. Mark C. Healey
Department: Biology

This study represents an effort to interrupt the life cycle of *Eimeria tenella*, the parasite that causes cecal coccidiosis in chickens, with a panel of 12 gamont-specific monoclonal antibodies (Mabs). To facilitate Mab screening, it was first necessary to develop a cell culture-adapted strain of *E. tenella* that was capable of producing large numbers of parasites in vitro. This was done by alternate passage of *E. tenella* (field strain 80) from primary chicken kidney cells (PCKC) to white Leghorn chickens and back to PCKC. This alternation was repeated through 12 such passages. As a result, we have developed a cell culture-adapted strain of *E. tenella* that produces over 280% more oocysts in vitro than the original parent strain, and over six times as many oocysts as reported by other investigators. Of the 12 Mabs evaluated, Mab HD8

had the greatest inhibitory effect by significantly reducing ($P<0.05$) the number of *E. tenella* oocysts produced in cell culture by 61% compared with untreated controls. Western blot analysis revealed that the target antigen for Mab HD8 had a molecular mass of 71 and 36 kilodaltons, respectively, when run under nonreduced and reduced conditions. Subsequent evaluation of soluble and membrane-associated proteins indicated that the target antigen for Mab HD8 was membrane-associated. In vivo studies are now required to validate the efficacy of this antigen in a subunit vaccine.

(72 pages)

LITERATURE REVIEW

Coccidiosis

Background. Coccidiosis is a disease caused by infection with protozoan parasites belonging to the subclass Coccidia (43). Coccidiosis poses a significant threat to the livestock industry on a world-wide basis, especially where animals are raised in confinement. This disease is particularly devastating to the poultry industry because of the pathogenic coccidians that infect birds and the extreme crowding that occurs in large-scale poultry operations. The most economically important parasites of this group belong to the genus *Eimeria*, with the single most pathogenic poultry coccidian being *Eimeria tenella* (42). Coccidiosis has been a major problem for producers since large-scale poultry farms came into existence.

Since the advent of anti-coccidial drugs, severe poultry losses have been less frequent. However, the cost of controlling this disease continues to increase. For example, in 1965 the USDA estimated that American poultry producers lost over 35 million dollars to avian coccidiosis (42). By 1985, estimated losses had risen to 300 million dollars in the United States alone (8). In 1992, the world-wide monetary cost of poultry coccidiosis was estimated to be near 2 billion dollars a year (3), thus making coccidiosis the most economically devastating

parasitic disease of poultry (51).

Life cycle. Parasitism by *E. tenella* begins with the ingestion of a fully mature, sporulated oocyst (Fig. 1). Subsequently, through the mechanical action of the gizzard, the oocyst releases four sporocysts. Initiated by the actions of chymotrypsin, bile, and carbon dioxide, each sporocyst then releases two sporozoites, resulting in a total of eight sporozoites being liberated from each ingested oocyst (43-45, 48). The sporozoites then pass through the gastrointestinal tract of the bird until they reach the ceca. Sporozoites may then either directly penetrate the intestinal epithelial cells or migrate via macrophages (42, 48), which carry them into the glandular epithelial cells of the crypts. The sporozoites ultimately locate between the nucleus and the basement membrane of the host cells and become trophozoites. Trophozoites are actively feeding vegetative stages which begin a series of asexual divisions (multiplying by a process known as schizogony) to form schizonts. Each schizont contains up to 900 merozoites (2-4 μm long). Upon being released from the schizont, each merozoite is capable of infecting a surrounding cell and once again undergoes a second round of schizogony, producing approximately 250 additional merozoites. Some of these merozoites go through yet another generation of schizogony. However, the majority

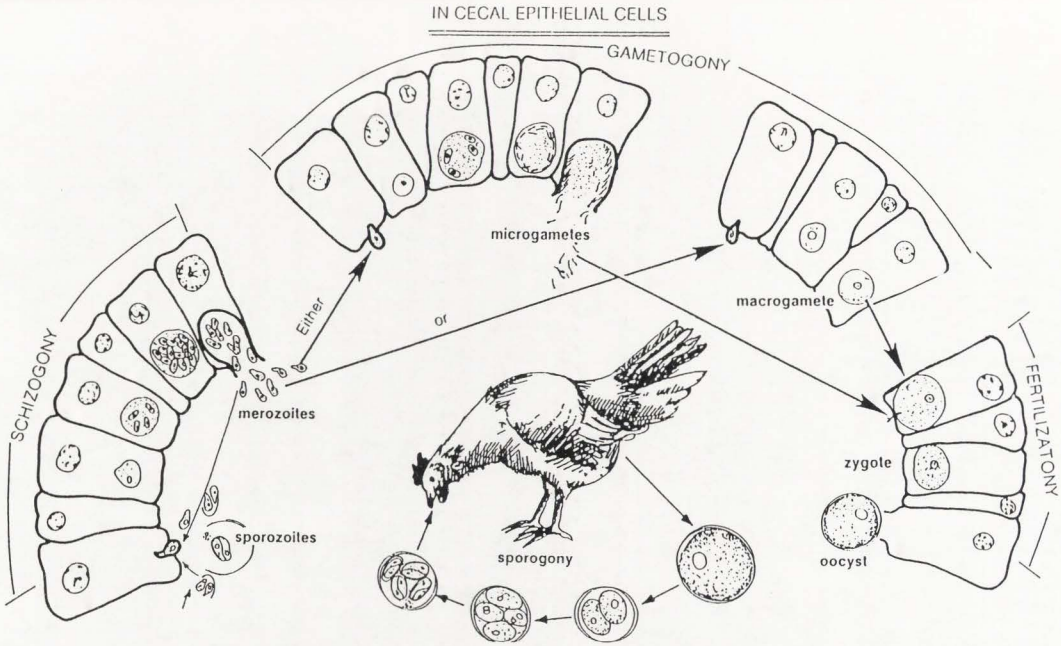


Fig. 1. The complete life cycle of *Eimeria tenella*.

undergo gametogony and develop into the sexual stage of the parasite known as the gamont; microgamonts represent the male stage and macrogamonts represent the female stage. After fertilization by a microgamont, the macrogamont forms a thickened wall around the zygote, which then develops into an oocyst. The oocyst is released from the host cell and passes out with the feces. The oocyst then sporulates in the presence of oxygen, moisture, and heat. Following sporulation, the oocyst becomes infective should it be ingested by a suitable host (42, 48).

Pathology. The life cycle of *E. tenella* causes significant intestinal damage in both the sexual and asexual stages of development. This damage results in bloody diarrhea, sloughing of patches of intestinal epithelial cells, and extensive cellular damage in the ceca. Damaged tissue is often infected by the intestinal flora of the bird, resulting in secondary bacterial infections which exacerbate the effects of the parasitic infection (2). Mortality and morbidity due to coccidiosis are often devastating to poultry producers. Mild infection results in weight loss and growth retardation. Severe infections may cause hemorrhage, emaciation, anemia, and death. Flock mortality up to 20% has occurred within a period of 2 to 3 days following infection by *E. tenella* (42). Chronic blood loss may result in exaguination and death by 5 to 7 days postinfection, with the hematocrit and

erythrocyte count of infected birds decreasing by up to 50% by days five and six of the infection (42). Clinical signs include general unthriftiness, lethargy, huddling, and ultimately death (6, 44, 45).

Control. The first successful control measure for *E. tenella* was announced in 1935 at the annual meeting of the American Society of Parasitologists when inorganic sulfur was shown to be an effective drug for treating coccidiosis. This discovery eventually led to the use of sulfonamides as anti-coccidial chemotherapeutics. Several drugs are now available to control coccidiosis in chickens. In fact, an almost direct correlation between growth in the broiler industry and the introduction of various anticoccidials has been demonstrated (44). These new drug discoveries proved to be very helpful in controlling coccidiosis for a limited time. However, with the advent of each anti-coccidial drug, the emergence of drug-resistant strains of *E. tenella* soon followed. In many cases, the emergence of these drug-resistant strains appeared within weeks to months of the drug's introduction into the field (43). The introduction of monensin in 1971 and the advent of other polyether ionophores showed great promise in controlling coccidiosis (65). This family of drugs has been a mainstay of the poultry industry in its attempt to control coccidiosis. Recently, however, researchers have demonstrated parasite

resistance to many of the drugs which are widely used in current coccidiosis control programs (65).

In addition to the difficulties presented to the poultry industry by drug-resistant parasites, the enormous cost of clearing new anti-coccidial compounds has become prohibitive. It is now generally acknowledged among pharmaceutical producers that the task of securing approval for a new drug is more difficult than its initial discovery, manufacture, and development (8). Collectively, these factors form a scenario in which more and more coccidiostat-resistant strains of *Eimeria* are appearing while less money is being invested to combat these emerging strains (8).

Vaccine potential. An alternative avenue of control for cecal coccidiosis would be the use of the bird's own immune system. It is well documented that birds produce protective immunity to coccidiosis after infection with viable parasites (3, 25, 40, 43). The live vaccine Coccivac® was developed with this strategy in mind. However, there are several drawbacks to using live vaccines in chickens. For example, live vaccines are expensive to produce and have a relatively short shelf-life (9). Live vaccines are designed to produce subclinical infections. However, any infection decreases feed efficiency and weight gain (43). Lastly, live vaccines may introduce new strains and species of coccidian parasites into a flock (49).

Oocysts passed by vaccinated birds may accumulate, becoming a constant source of infection for new birds, should vaccination ever be discontinued (33). Because of these concerns, it is doubtful that a live vaccine could fulfill current market requirements (22).

A subunit vaccine, consisting of protective parasitic antigens, would resolve many of the problems associated with a live vaccine (8, 22, 40). Ideally, a subunit vaccine would be stable, have a longer shelf-life, would not cause the weight loss associated with live vaccines, and would not contaminate pens with viable oocysts. Development of subunit vaccines has been attempted with sporozoites (1, 8) and merozoites (29).

Recombinant vaccine potential. Several approaches to the development of an effective subunit vaccine have been used (6). The most widely practiced method of developing coccidial vaccines is an empirical approach in which monoclonal antibodies (Mabs) are produced against parasitic antigens (3). These Mabs are evaluated to determine whether they inhibit the parasite's life cycle. Those Mabs demonstrating efficacy are further evaluated by identifying their target antigens. Complementary DNA's are then cloned and inserted into the genome of bacteria through the use of bacteriophages. The bacteria then produce the desired parasite protein antigen; these

recombinant antigens are then used in the subunit vaccine (8, 10, 22, 59).

Both humoral and cell-mediated immunity have been shown to be important in naturally-acquired immunity to *Eimeria* species (60). Hence, the most effective antigens would presumably include both T-cell and B-cell immunogens. However, it has been shown that an antibody response alone is insufficient to clear eimerian infections, and that while infection with one species of *Eimeria* does not confer immunity to other species, there is an abundance of cross-reactive T-cells that recognize different species of *Eimeria* (39). This indicates that the combination of T and B-cells functioning in concert is imperative in successful protection against poultry coccidial infections. Several of the subunit vaccines being explored today incorporate both B and T-cell immunogens (4, 42). Antigens that share B and T-cell epitopes have been shown to produce promising results in vivo (3-5).

Each of the subunit vaccines evaluated to date has provided partial protection in vaccinated birds, but none has afforded total protection against coccidiosis (5, 8). For this reason, the concept of incorporating antigens from several stages of the parasite's life cycle is considered essential (22). If the life cycle of *Eimeria* is to be inhibited, antigens from both the asexual and sexual stages of the parasite are vital. Such antigens would be good

candidates for vaccines in that both sexual and asexual stages are known to be very antigenic (40, 62). Through a multivalent subunit vaccine approach, it is believed that the sum of the cumulative partial effects of each immunogen would produce almost total protection against *Eimeria* (5).

Recently, a panel of 13 Mabs was produced against the sexual stages (gamonts) of *E. tenella* (30). These Mabs could theoretically be used to isolate parasite antigens capable of inducing protective immunity in chickens and hence be utilized in the development of a subunit vaccine. In a pilot study, one of these Mabs (GD9) was shown to reduce oocyst shedding and mitigate cecal lesion scores when administered per os to infected chickens (31).

Cell Culture of Coccidians

Background. The first coccidian to be grown in vitro was *E. tenella*; this eimerian was first grown in Madin-Darby bovine kidney (MDBK) cells (37). Researchers observed that parasitic sporozoites were able to penetrate MDBK cells and complete one cycle of schizogony, but were unable to progress further in their life cycle. In 1970, working under the premise that species-specific parasites would grow better in cells obtained from natural hosts, Doran successfully grew *E. tenella* to the completion of its life cycle using primary chick kidney cells (PCKC)(11). Although Doran surmounted a major obstacle in successfully

culturing the parasite to the completion of its life cycle in vitro, his yields were relatively poor. One experiment using an innoculum of 500,000 sporozoites yielded only 16 oocysts (46). Low yields of *E. tenella* oocysts produced in vitro have continued to be a major stumbling block in efforts to better understand the biology and biochemistry of this parasite.

Host cell lines. Since the first report of in vitro development by Patton (37), researchers have used a number of different cell lines to culture *E. tenella* and improve oocyst yields. Chick kidney cells have repeatedly been shown to support the growth of *E. tenella* from sporozoite to oocyst (13-15, 28). Likewise, embryonic chicken kidney cells have successfully supported parasite development to the oocyst stage, but have been less efficient than nonembryonic kidney cells (11, 53). Quist et al. (41) demonstrated that kidney cells from a resistant line of Auburn White Leghorn chickens produced fewer oocysts in culture than a susceptible line from this same strain of chicken. Since propagation of parasites in vitro eliminates the influences of the humoral and the cell-mediated immune responses, these results demonstrate that not only immune mechanisms, but also host cell genetic influences affect in vitro growth and development of *E. tenella*.

Bovine kidney and embryonic bovine tracheal cells

have been shown to sustain the growth of *E. tenella* through one cycle of schizogony (19, 37). Primary chicken fibroblast and quail fibroblasts were also shown to support the parasite through a single round of schizogony (11). Primary turkey kidney cells have been used to grow *E. tenella* through two cycles of schizogony, and partridge kidney cells have supported the parasite growth and development to the gamont stage. Primary pheasant kidney cells have successfully supported the life cycle of *E. tenella* to the oocyst stage; however, oocyst yields were never as high as in PCKC (21). Researchers have noted that after 4 h postinfection with sporozoites, partridge, chicken, and pheasant primary kidney cells all yielded identical infection rates, yet sporozoite development was much better in the chicken cells (21). These results demonstrate that development of *E. tenella* in cultured cells is dependent upon the phylogenetic relationships of the specific host cells employed (15).

Environmental conditions. The basal temperature of the chicken is 41°C, and it appears that *E. tenella* grows optimally at this temperature (52). However, *E. tenella* is routinely cultured at 40.5°C (54). This one-half degree difference is used as a buffer in order to protect the parasites from temperature fluctuations above 41°C, at which parasite development rapidly deteriorates (52).

Normal atmospheric levels of oxygen range from 18-

21%, but the lumen of the gut is anaerobic. Theoretically, anaerobic cell culture conditions would be more amenable to the survival of extracellular parasitic stages, and atmospheric levels of oxygen potentially detrimental to the host cell. However, host cells are oxygen dependent for growth and respiration. In an effort to find the oxygen tension that is best suited to supporting parasitic growth within host cells, researchers have attempted to manipulate a variety of environmental conditions. For example, Tilley and Upton (56) observed that under aerobic conditions, *Eimeria nieschulzi* could survive for only 2 days in cell culture. When this parasite was grown under reduced oxygen conditions, it consistently underwent four cycles of schizogony and survived at least 8 days. Strout and Schmatz (54) showed that at oxygen levels as low as 0.1%, host cell division was reduced, but schizogony increased by over 60%. They also showed a two-fold increase in coccidian gametogony in reduced oxygen cultures.

Cell culture microenvironment. Doran noted that PCKC aggregates seemed to be much better suited for growing *E. tenella* than a simple monolayer of single cells (11). He also noted that parasites developed focally within cell cultures; i.e., one area of the culture may be heavily infected, while adjoining areas may be totally devoid of parasites. The fact that *Eimeria* infections occur in sporadic patches throughout the cell culture makes

quantitation of the parasite difficult because accurate enumeration cannot be made simply by observing a set number of random fields under the microscope. This requires that the entire cell monolayer be observed and evaluated. Parasitic growth in sporadic patches also suggests that several generations of a more susceptible host cell may have accumulated in the cell culture flask or plate. Hence, if a subpopulation of host cells could be selected in which parasite development occurred, it should be possible to increase the number of parasites grown in vitro. At the same time, quantitating parasite growth and development would be facilitated in that a number of random fields could be counted to obtain an accurate estimation of the total number of parasites present (46).

To test the hypothesis that certain clones within a population of cells would better sustain parasite growth, Schmatz (46) isolated 22 clones from a population of MDCK cells and tested each subclone for its ability to sustain the intracellular growth of *E. tenella*. Results ranged from less than 1.0% of cells infected in one clone to over 13.0% of the cells containing intracellular parasites in other clones. The parent cell line was shown to have just over 6.0% of the cells infected with *E. tenella*. These results suggested that the focal development of parasites may indeed be due to the accumulation of several

generations of more susceptible host cells within the cell culture flask.

Even though sporadic focal growth may be a result of parasites developing in the progeny of a single, more susceptible host cell, it is also conceivable that highly concentrated areas of the cell monolayer may create a microenvironment more conducive to parasitic growth (12). In cross titration studies in which the ratio of parasites to host cells was measured, it was found that *E. tenella* grew better in more confluent monolayers (47). Researchers have also noted when culturing *Toxioplasma gondii* that the number of host cells present influences parasite performance, suggesting that the three-dimensional microenvironment of the cell culture plays an important roll in the ability of eimerian parasites to complete their life cycles in vitro (12).

Although many researchers have studied the effects of manipulating cell culture conditions extensively, they have not been able to apply their knowledge to greatly increase the number of oocysts grown in cell culture (11, 28). While several papers have been published on manipulating cell culture conditions (7, 28, 32, 41, 57, 63), there seems to be a dearth of information concerning the possibility of altering the parasite to increase its performance in vitro.

In vitro quantitation of parasite growth and development. Quantitation of parasitic growth in early experiments involving *E. tenella* cultivation was often accomplished by simply fixing and staining cell monolayer-containing coverslips (18). The number of parasites within the host cells could then be visually counted or estimated by counting a predetermined area on the coverslip (13, 53).

Further studies were done in which antibodies against second generation merozoites were used in an enzyme-linked immunosorbent assay (ELISA) to quantitate in vitro development of *E. tenella* (36). The ELISA is best suited for evaluating anticoccidial drugs that target parasitic stages produced during first generation schizogony.

Incorporation of ^{3}H-uracil has also been used to quantitate the in vitro development of several different types of intracellular parasites (47). This is possible because mammalian cells possess minute amounts of uracil phosphoribosyl transferase, whereas eimerians have substantial levels of this enzyme. This enzyme allows for specific labeling of the parasite, while the amount of nonspecific ^{3}H-uracil uptake by the host cell is kept to a minimum. By employing this method, it is possible to correlate the amount of ^{3}H-uracil uptake with the amount of in vitro growth of the parasite (38, 58).

Automated methods such as uracil uptake and the ELISA are fast and relatively easy, both being desirable if an

extremely large number of samples are to be processed (36, 47). However, these methods also have several limitations. Uracil uptake is not sensitive to different stages of the parasite because uracil is taken up any time that DNA is being actively transcribed (47). The ELISA method can detect the presence of any stage of the parasite, but is only reliable if the desired stage is extracellular and if there are sufficient numbers of the stage present in the cell culture (36).

Manual methods of parasite quantitation, such as fixing and staining or simply direct counting, are much more laborious than the aforementioned methods. Fixing and staining makes it possible to easily distinguish between different stages in the parasite's life cycle. However, if the parasite is extracellular at the time of staining, it may be washed away before it is counted. For stages such as oocysts that may be intracellular as well as extracellular, it is preferable to count them directly, omitting fixing or staining which may cause the loss of extracellular stages.

Molecular Weight Determination of Parasitic Antigens

Background. Molecular characterization of macromolecules is of particular importance in gaining an understanding of protein biochemistry, size, and chemical

composition (59). Evaluations of this type are also useful in discerning differences between closely related species or strains of microorganisms. For example, molecular characterization has been used to determine protein differences between parasitic stages (64). Gleaning an understanding of these differences could facilitate an appreciation of parasite biochemistry and host-parasite interactions.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis. Gel electrophoresis is among the most powerful and conveniently used methods of macromolecular separation (10). Molecular separations are based on gel filtration as well as the electrophoretic mobility of the molecules being separated (55). In polyacrylamide gel electrophoresis (PAGE), gels are made by the free radical-induced polymerization of acrylamide and N',N'-methylenebisacrylamide (59).

Detergents are ampipathic molecules that strongly denature proteins (59). Sodium dodecyl sulfate (SDS) is a commonly used, tenaciously binding detergent which assists in denaturing proteins, thus causing them to assume a rod shape (10). This reduces the problem of different density proteins migrating at different speeds due to the gel filtration aspect of electrophoresis. Sodium dodecyl sulfate also imparts a large negative charge that masks the protein's intrinsic charge. This phenomenon tends to cause

SDS-treated proteins to have identical charge-to-mass ratios and similar shapes (24).

Western blotting. The use of SDS-PAGE is an effective way to separate proteins of various molecular weights (10). However, once these proteins have been separated, identifying individual proteins may still be a formidable task. Through the use of Western blotting, molecules that have been separated on the gel are then transferred to a nitrocellulose membrane (26). The nitrocellulose is then bathed in antibody. If the antibody is directed against a protein or some other molecule present on the membrane, hybridization will occur and the antigen-antibody complex can then be identified with a labeled reagent that reacts with the complex (26). Western blotting has been used to identify the molecular weight of individual proteins, including several which are an intrinsic part of parasitic gamonts (61, 62). This technique is an important method to partially characterize antigens, because it helps to determine target antigen size and establish the existence of an antigen-antibody interaction (10).

OBJECTIVES

The goal of this research is to ultimately determine which Mabs in a previously produced panel of 13 will prevent *E. tenella* from completing its life cycle in vitro. Those Mabs demonstrating significant inhibition could then be used to identify and isolate antigens that could be evaluated in a subunit vaccine for use in chickens at risk of cecal coccidiosis. Specific research objectives are as follows:

1) Develop an improved cell culture system in which *E. tenella* can be successfully adapted to grow and reproduce. This will include adapting a field isolate of the parasite (strain 80) to the improved cell culture.

2) Employ the parasite-adapted cell culture system to evaluate a previously produced panel of Mabs for their ability to interrupt the life cycle of *E. tenella*.

3) Identify antigens to which each Mab binds by Western blot assays. Determine the molecular weight of target antigens and ascertain if the antigens are derived from the membrane-associated or water soluble fraction of the gamont.

MATERIALS AND METHODS

Adapting a Field Isolate (Strain 80) of *E. tenella* to Cell Culture

Source of oocysts. The field isolate of *E. tenella* (strain 80) used to inoculate chicks was kindly provided by Dr. Harry D. Danforth. Oocysts were stored in 2.5% potassium dichromate ($K_2Cr_2O_7$) at 4°C. Before use, oocysts were washed twice in distilled water to remove the potassium dichromate.

Source of chickens. Fertile eggs of White Leghorn chickens were obtained from the Utah State University (USU) poultry farm and hatched in a standard incubator. The resulting chicks were housed in the USU Laboratory Animal Research Center and maintained in a Duo Flo II chamber (Biochem Lab Products Inc., Maywood, N.J.) at 22-27°C and 28-35% relative humidity. Chicks were fed 20% laying mash (Trenton Feed Cooperative, Trenton, Utah) until they were killed by CO_2 asphyxiation.

Inoculation of chickens. Chicks were individually inoculated per os at 2 to 4 weeks of age with 30,000 sporulated oocysts of *E. tenella* which had been previously diluted in distilled water.

Cell culture preparation. Kidneys were obtained and pooled from two 1- to 4-week-old chicks following CO_2 asphyxiation. The kidneys were removed using sterile

technique, transferred to a 50-ml centrifuge tube, washed twice in Hank's Balanced Salt Solution (HBSS), and minced into small pieces (about 1 mm^3) with scissors. Pieces of kidney tissue were placed in a beaker with 50 ml of preheated 0.25% (w/v) trypsin (DIFCO, Detroit, Mich.) solution consisting of 0.25 g of 1:250 trypsin dissolved in 100 ml of HBSS. Kidney pieces were then stirred slowly for 5 minutes (min) at 37°C, allowed to sediment, and the supernatant discarded. Fifty milliliters of preheated fresh trypsin solution were added to the pellet and slowly stirred for 15 min at 37°C. The supernatant was decanted and saved before the PCKC settled out. Five milliliters of fetal bovine serum (FBS) (Hyclone Laboratories, Logan, Utah) were immediately added to neutralize the remaining trypsin. Primary chick kidney cells were then centrifuged at 200 x g for 5 min and washed twice with HBSS. The PCKC were then diluted 1:10 with growth medium consisting of 89% RPMI-1640, 10% heat inactivated FBS, and 1% Antibiotic Antimycotic Solution (Sigma Chemical Co., St. Louis, Mo.). A PCKC suspension of 0.7 ml was added to 100 ml of growth medium. One milliliter of medium was used to seed each well in a 24-well polystyrene cell culture plate (Corning, Corning, N.Y.) containing a 13-mm diameter Thermanox coverslip in each well (Nunc Inc. Naperville, Ill.). The plates were incubated at 40.5°C in 5% CO_2 for 48 to 60 h until the cell culture reached 80% confluency.

Inoculation of the cell culture. Fresh oocysts were washed in 40% bleach for 15 min in an effort to kill contaminating bacteria. Oocysts were then washed in HBSS approximately five times until the smell of bleach could not be detected. Oocysts in a volume of 3 to 5 ml were placed in a 50-ml centrifuge tube containing 5 ml of glass beads (Sigma). The tube was vortexed for 30-60 seconds, until 80% of the oocysts had ruptured and released their sporocysts. The glass beads were washed with 50 ml of HBSS. Oocysts suspended in HBSS were centrifuged for 10 min at 1875 x g and the supernatant decanted. The pelleted sporocysts were added to 50 ml of excystation solution consisting of 0.375 g taurodeocycholic acid (Sigma) with 0.125 g of trypsin (DIFCO) in HBSS and incubated at 40.5°C in 7.5% CO_2 for 40 min or until 80% excystation had occurred. When excystation was completed, sporozoites were passed through an 11-µm filter (Whatman International Ltd., Maidstone, England) to remove any remaining intact oocysts and sporocysts. Sporozoites were then counted using a hemocytometer, washed once in HBSS, and diluted in growth medium to a concentration of 2-10 X 10^4 per ml. One milliliter of medium was then added to each monolayer of PCKC in cell culture wells. The medium was changed on days one, three, and five postinfection by aspirating approximately three fourths of the old medium from each well and adding 1 ml of fresh preheated medium.

Parasite quantification. On day seven postinfection, coverslips were carefully removed from culture wells and placed cell-side up on a 1x3 glass microscope slides. A clean glass coverslip was placed over each cell monolayer and the oocysts were counted by scanning the entire coverslip at X100 magnification using brightfield microscopy.

Adapting *E. tenella* to cell culture. Oocysts of *E. tenella* (strain 80) were excysted and the resulting sporozoites were used to infect PCKC cultures. Seven days postinfection, oocysts which had developed in PCKC culture were harvested and sporulated at room temperature for 5 days. Following sporulation, oocysts were used to infect 2-week-old chicks. Seven days postinfection, chicks were killed and their ceca removed. The cecal contents containing oocysts were placed in 2.5% potassium dichromate for 5 days to allow for oocyst sporulation. Oocysts were then used to infect a second PCKC culture. These oocysts were referred to as the F1 generation since the parasite had passed through PCKC culture once previously. After passage through PCKC culture a second time, the oocysts were harvested and again used to infect chicks. Oocysts collected from these chicks were referred to as the F2 generation. Alternating from chicken to PCKC culture and back to chicken was repeated 12 times.

At the same time that PCKC culture-adapted parasites

were being used to infect chicks, oocysts of *E. tenella* which had never been exposed to PCKC culture also were used to infect chicks. The cell culture-naive oocysts were then used as controls.

Optimizing the percentage of FBS. In an attempt to determine the optimum percentage of FBS for use in the PCKC culture system, experiments were performed using 5%, 7.5%, and 10% FBS. Pilot studies in our laboratory had previously shown that excessive amounts of FBS may cause PCKC cultures to peel away from the glass coverslips, while inadequate amounts of FBS resulted in low oocyst yields.

In Vitro Evaluation of Monoclonal Antibodies (Mabs)

Production of Mabs. Hybridomas producing Mabs originally produced by Larsen (30) were grown in 89% DMEM, 10% FBS, and 1% Antimycotic Antibiotic Solution in an atmosphere of 5% CO_2 and 100% humidity. Hybridomas were maintained until supernatants became acidic (yellow). Supernatants containing Mabs were then collected and stored at 4°C in 0.02% NaN_3 for future use. Mabs used as controls (sham Mabs) were grown under identical conditions, but directed against an outer membrane protein of an *Actinobacillus* species. Sham Mabs were of a similar isotype (IgG) as the anti-gamont Mabs and were originally produced by Healey et al. (27). Sham Mabs were determined not to cross react with *E. tenella* antigens.

Concentration and purification of Mabs. A six inch glass column was packed with 5 ml of protein G-coated Sepharose beads (Pharmacia LKB Biotechnology, Piscataway, N.J.). The column was equilibrated with 50 ml of starting buffer which consisted of 0.05 M phosphate, 0.015 M NaCl (pH 8), 6.7 g $Na_2HPO_4 \cdot H_2O$, 0.38 g NaH_2PO_4, and 8.76 g NaCl pH adjusted to 8.0 using NaOH to a final volume of 1 L. One hundred and fifty to 200 ml of spent supernatant were then passed through the column three times. The column was flushed with starting buffer until extraneous proteins were washed out. The eluting buffer consisted of 0.05 M glycine and 0.15 M NaCl, with the pH adjusted to 2.5 using HCl. The flow-through was then collected in 1-ml aliquots in tubes containing one drop of 1 M Tris-HCl buffer (pH 9). Following the elution of the Mabs, the column was re-equilibrated to pH 8 with starting buffer containing 0.02% NaN_3 and stored at 4°C. The Mab concentration in each of the aliquots was determined by using a total protein assay kit (Pierce, Rockford, Ill.). Monoclonal antibody purity was verified by using a 12% SDS-PAGE gel followed by silver staining. Aliquots of Mab were stored at -20°C for future use.

Use of Mabs in cell culture. Immediately before use, each Mab was thawed and passed through an Acrodisc 0.2-µm filter (Gelman Sciences, Ann Arbor, Mich.). Monoclonal antibodies were then diluted with growth medium

to 30 µg/ml (high concentration) and 5 µg/ml (low concentration). Control and experimental (infected) cell culture wells containing PCKC received the same concentration and volume of each Mab. Monoclonal antibodies were added to infected cell culture wells on days three and five postinfection.

At the end of the 7-day life cycle, coverslips containing monolayers of PCKC were removed from the cell culture wells and placed cell-side up on glass 1x3 microscope slides. A clean glass coverslip was then placed over each monolayer and the oocysts were counted by scanning the entire coverslip at X100 magnification, using brightfield microscopy. The numbers of oocysts in the wells containing gamont-specific and sham Mabs were statistically compared using an unpaired Student's *t* test.

Partial Characterization of Mabs

Gamont collection. At 5.5 days (134 h) postinfection, chickens were killed, the infected ceca collected, and the gamonts of *E. tenella* harvested by using a modification of the methods reported by Larsen (30) and Larsen et al. (31) and Wallach et al. (62). Briefly, the ceca were placed in a 100 X 15-mm style disposable polystyrene petri dish, opened lengthwise, and the contents flushed out with SAC buffer (170 mM Tris-HCl, pH 7.0, 10 mM glucose, 5 mM $CaCl_2$, 1 mM phenylmethyl sulfonyl fluoride). The cecal mucosal

cells were then removed by gently scraping the luminal surface with a scalpel. Infected cells were lysed to release intracellular gamonts by homogenizing the tissue suspension in a stomacher (Tekmar Company, Cincinnati, Ohio) for approximately 30 seconds. Cellular debris and remaining tissue were removed by passing the solution through four layers of cotton gauze. The gamont-containing follow-through was then collected. Gamonts were further purified using a Percoll density gradient (Sigma) with Percoll concentrations of 40, 30, 20, and 10% in a 50-ml tube. The tube was centrifuged at 2,700 x g for 40 min (31). Gamonts were normally found in the interface between 20% and 30% percol concentrations.

Gamont antigen preparation. Following purification, parasitic gamonts were washed twice in SAC buffer without BSA and frozen at -60°C to lyse the gamonts. The gamont suspension was centrifuged at 7,000 x g for 10 min and the supernatant collected. This fraction was later used as the protein preparation for the analysis of soluble gamont antigens. The resultant gamont pellet, presumably made up of insoluble membrane components, was placed in a nonionic detergent solution consisting of 1% *n*-octyl-ß-D-glucopyranoside diluted in SAC buffer. The gamont suspension was incubated for 1 h with slow stirring at 37°C and centrifuged at 7,000 x g for 10 min. The supernatant was dialyzed against 4 liters of PBS twice over a 24-h

period and centrifuged at 7,000 x g for 20 min at 4°C. The protein concentration of these membrane-associated proteins was then determined by means of a Pierce total protein assay (Pierce). Supernatant was used as the antigen preparation for gel electrophoresis (27). Control antigen was prepared by the same method, except only the mucosa of noninfected chicks was used and the stomaching and Percoll purification steps were omitted.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a MINI-PROTEAN II (Bio-Rad) gel electrophoresis apparatus. Results obtained with the MINI-PROTEAN II were confirmed by running a large gel on a PROTEAN II xi Cell (Bio-Rad). The sample run in each lane consisted of the protein antigen preparation described above (section on gamont antigen preparation) at a concentration of 1 mg/ml diluted 1:4 with sample buffer. The sample buffer consisted of 62.5 mM Tris-HCl (pH 6.8) 10% glycerol, and 2% SDS. Five percent ß-Mercaptoethanol (BME) was added to those samples that were reduced. ß-Mercaptoethanol was omitted from those samples that were nonreduced.

Western blots. Following SDS-PAGE, separated antigens were electroblotted to nitrocellulose sheets, pore size 0.2 µm (Schlecher and Schuell, Keene, N.H.) using a Trans-Blot

Cell (Bio-Rad). Antigen transfer was done for 15 h at 15 volts and an amperage limit of 0.22.

Blotting results were visualized by cutting the nitrocellulose sheets into individual strips and placing each strip into a plastic tray. Each nitrocellulose strip was then blocked with 3% BSA in Tris buffered saline (TBS consisting of 2.42 grams Tris and 7.9 grams NaCl per liter of distilled water and pH adjusted to 7.4) for 1 h at 30°C under constant agitation. The strips were then washed three times with TBS Tween (TBS with 0.1% Tween 20) for 10 min per wash. The nitrocellulose strips were probed with spent hybridoma supernatant containing Mabs for 1 h and washed as described above. Secondary antibody consisting of anti-mouse IgG (whole molecule) peroxidase-conjugate (Sigma) diluted 1:2,000 in TBS was added, incubated for 1 h, and washed as described above. Horseradish peroxidase substrate reagent was then added (Bio-Rad). After approximately 20 min of color development, the strips were washed in distilled water for 10 min and air dried. Antigenic molecular weights were then calculated by comparing with nitrocellulose strips in which protein standards had been run. After antigen transfer, those strips containing molecular weight markers were stained in 0.2% Ponceau S in 1% acetic acid for 1 h and destained in 1% acetic acid. The molecular weights of the gamont antigens were determined by comparing migration distances

of the bands with the protein standards. Along side each lane of gamont antigen was a lane of noninfected cecal antigen prepared as described earlier. Controls were also run in which the primary antibody was omitted but secondary antibody and substrate were used. Reduced and nonreduced antigens were used to determine if epitopes were located on a protein consisting of subunits and to determine if antigenisity was destroyed by denaturation or disulfide bond reduction. Antigens from sporozoites, merozoites, and oocysts of *E. tenella* have been shown not to cross-react with any of the anti-gamont Mabs within our test panel (31).

RESULTS

Adapting *E. tenella* to Cell Culture

Primary chick kidney cell cultures inoculated with *E. tenella* sporozoites were observed to support all stages of parasitic development. Intracellular sporozoites were observed infecting PCKC within minutes to hours of inoculation (Fig. 2). Both first- and second-generation schizonts were seen within PCKC; mature first-generation schizonts first appeared after 36 h postinoculation (PI) (Fig. 3). First-generation merozoites were observed free within the culture medium at 48 h PI (Fig. 4). Due to the asynchronous development of *E. tenella,* monolayers of PCKC contained numerous trophozoites (Fig. 5), developing schizonts and many free merozoites (Fig. 6). Gamonts appeared after 120 h PI (Figs. 7 & 8).

Oocysts normally began to appear on day six PI and could be seen developing through day eight PI (Fig. 9). Several second-generation schizonts were often seen developing within a single PCKC (Fig. 10). However, the number of sexual stages remained relatively small. Occasionally, up to several hundred oocysts could be seen in isolated areas of the PCKC monolayer (Fig. 11). Several times while observing the cell culture-adapted strain of *E. tenella,* multiple gamonts and oocysts were

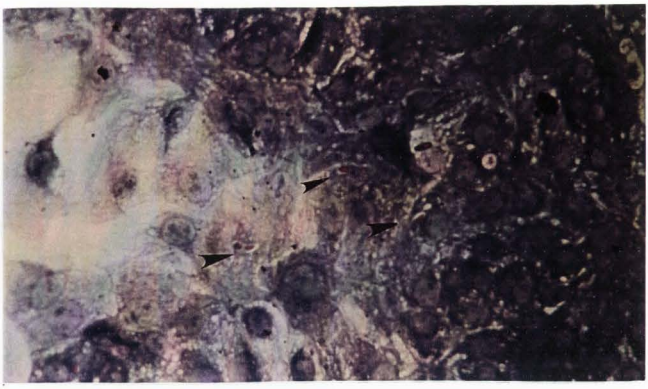


FIG. 2. Sporozoites of *Eimeria tenella* within primary chicken kidney cells (arrowheads) 4 h postinfection. Stained with aniline blue and acid fuchsin. Magnification, X400.

FIG. 3. Primary chicken kidney cell infected with a first generation schizont of *Eimeria tenella* containing first generation merozoites (arrowhead) at 36 h postinfection. Stained with Giemsa. Magnification, X400.

FIG. 4. First generation merozoites of *Eimeria tenella* free in the culture medium (arrowheads). Note the presence of a trophozoite beginning to develop (arrow) at 48 h postinfection. Stained with aniline blue and acid fuchsin. Magnification, X400.

FIG. 5. Early trophozoite of *Eimeria tenella* (arrow). Note one refractile body (red) of the sporozoite still visible within the developing trophozoite at 72 h postinfection. Stained with aniline blue and acid fuchsin. Magnification, X400.

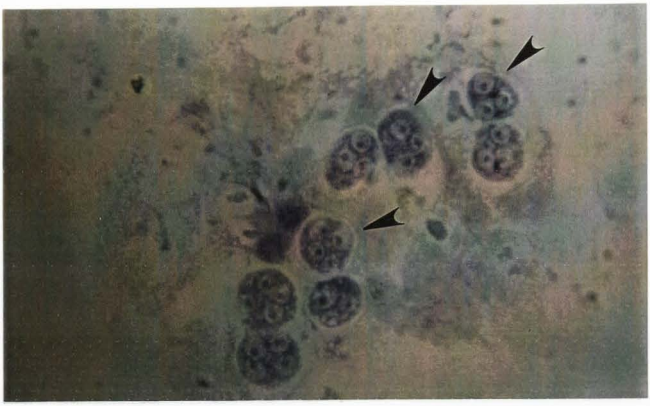


FIG. 6. Second generation schizonts of *Eimeria tenella* early in their development (arrowheads), containing three to four nuclei at 72 h postinfection. Stained with Giemsa. Magnification, X400.

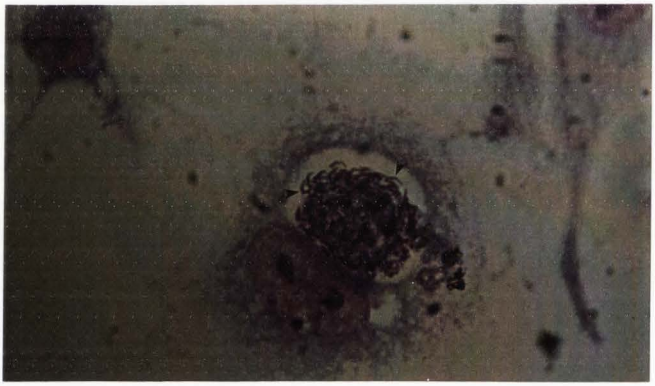


FIG. 7. Microgametocyte of *Eimeria tenella* growing within a primary chicken kidney cell at 7 days postinfection (arrowheads). Individual biflagulated microgamets can be seen within the parasitophorous vacuole. Stained with Giemsa. Magnification, X1000.

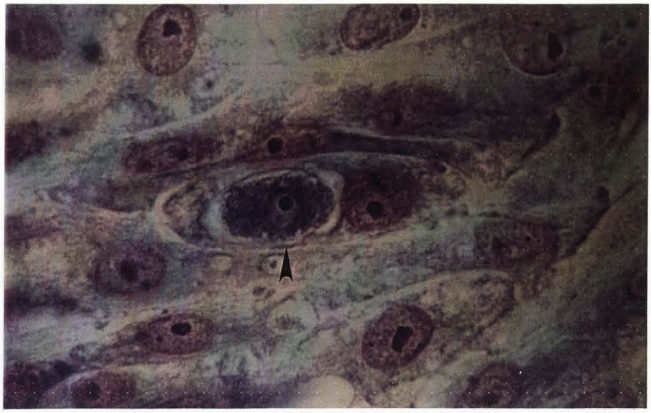


FIG. 8. Macrogamont of *Eimeria tenella* (arrowhead) growing within a primary chicken kidney cell at 7 days postinfection. Stained with Giemsa. Magnification, X400.

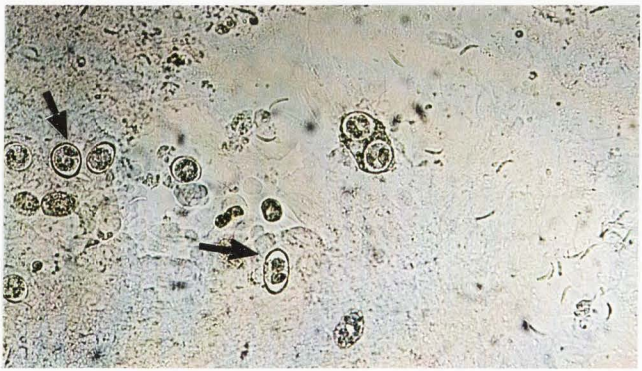


FIG. 9. Mature oocysts of *Eimeria tenella* in a primary chicken kidney cell culture 7 days postinfection (arrowheads). Magnification, X250.

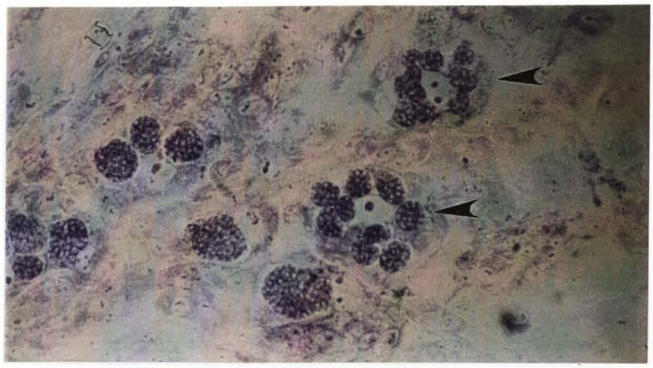


FIG. 10. Multiple second generation schizonts of *Eimeria tenella* (arrowheads). As many as ten schizonts can be seen within a single primary chicken kidney cell (PCKC). Note the greatly enlarged PCKC nucleus at six days postinfection. Stained with Giemsa. Magnification, X400.

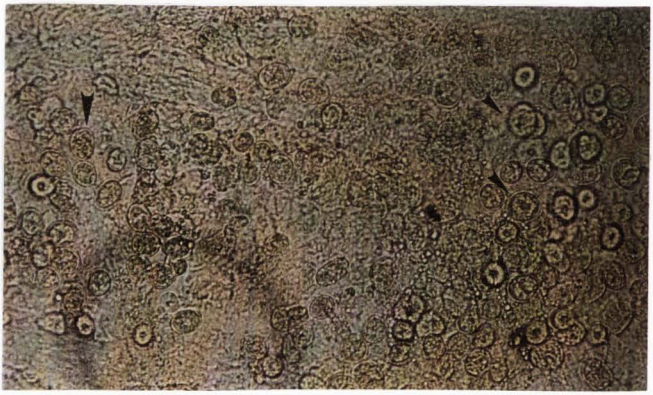


FIG. 11. More than 100 mature oocysts of *Eimeria tenella* in a primary chicken kidney cell culture (arrowheads) at 7 days postinfection. Magnification, X250.

seen developing within a single PCKC (Fig. 12).

The results of growing *E. tenella* in varying concentrations of FBS showed no appreciable difference in the number of oocysts produced (Table 1). All concentrations of FBS produced mean numbers of oocysts between 264-358, with no significant difference between them. Likewise, there was no significant difference between the numbers of wells that overgrew and peeled within groups receiving different concentrations of FBS.

When the F1 generation was compared with the F0 (parent) generation, an apparent increase in the number of oocysts produced in the cell culture-adapted strain of *E. tenella* appeared (Table 2). In the F1 generation, 29% more oocysts were produced compared with the F0 generation ($P = 0.479$). By the F2 generation, the percentage of oocysts produced exceeded that of the F0 generation by 261% ($P < 0.0001$). This increase in oocyst production continued throughout the experiment. By the F5 generation, oocyst output increased 280% over that of the parent generation ($P < 0.0001$).

In Vitro Evaluation of Mabs

Of the panel of 12 Mabs evaluated in vitro for their ability to inhibit the life cycle of *E. tenella* (1 of the original 13 Mabs produced was not viable when retrieved from storage; however, there was sufficient amount of

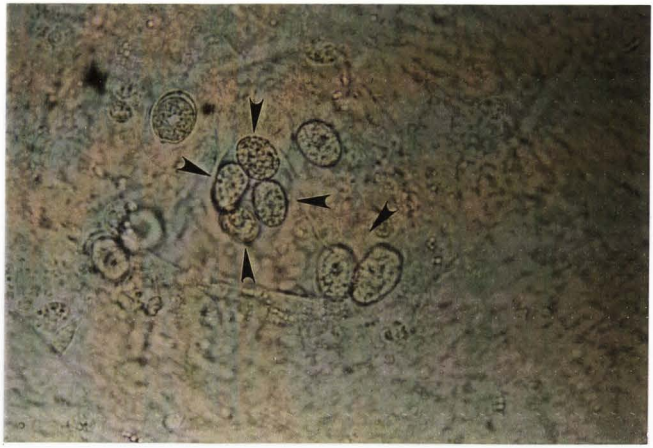


FIG. 12. Multiple oocysts of *Eimeria tenella* within a single primary chicken kidney cell (PCKC). Note the four oocysts within a single PCKC (arrowheads) and the two additional oocysts within another PCKC (arrowhead). Magnification, X250.

TABLE 1. The effect of varying concentrations of fetal bovine serum (FBS) on oocyst production

Group	Count[a]	Oocysts[b]	$SE_{\bar{x}}$
5% FBS	6	358	58
7.5% FBS	5	264	56
10% FBS	5	332	99

[a] Number of cell culture monolayers counted.
[b] Mean number of *Eimeria tenella* oocysts per cell culture monolayer.

TABLE 2. The ability of *Eimeria tenella* to adapt to PCKC following the repeated passage of oocysts between cell culture and chickens

Generation[a]	Oocysts[b]	SE$\bar{x}$	% Increase[c]	*p*[d]
F0	79.6	7.23		
F1	102.2	7.94	29%	0.479
F0	39.40	2.29		
F2	141.41	6.48	261%	0.0001
F0	28.44	1.71		
F3	103.35	4.69	267%	0.0001
F0	39.07	3.18		
F4	144.67	9.35	269%	0.0001
F0	66.60	7.14		
F5	251.40	22.02	280%	0.0001

[a] Cell culture generation, FO representing the parent strain and F1-F5 representing each subsequent passage through cell culture, respectively.

[b] Mean number of *E. tenella* oocysts per cell culture monolayer.

[c] Percent increase in the number of oocysts of the cell culture, adapted strain of *E. tenella* over the parent strain.

[d] Calculated by using Student's *t*-test.

supernatant to perform Western blotting as discussed latter), nine showed no effect at either concentration tested (Table 3). Two of the remaining three Mabs demonstrated an inhibitory effect at only one of the two concentrations tested. For example, Mab EA7 caused a significant reduction ($P < 0.047$) in the number of oocysts produced when used at 30 µg/ml. However, Mab EA7 was associated with a significant increase ($P < 0.034$) in the number of oocysts produced when used at 5 µg/ml. Results from use of monoclonal antibody EG3 were like those of Mab EA7. Only Mab HD8 significantly inhibited ($P < 0.05$) the number of oocysts produced when employed at two concentrations (24 and 35 µg/ml). However, when tested at 2.4 µg/ml, Mab HD8 had no inhibitory effect.

Partial Characterization of Mabs

Target antigens were identified in Western blots for all Mabs evaluated. However, 12 of the 13 Mabs reacted only with nonreduced antigens. Only Mab HD8 reacted with both reduced and nonreduced antigens. All Mabs reacted with nonreduced antigens having molecular masses of approximately 71 Kd (Fig. 13). Monoclonal antibody HD8 reacted most strongly with reduced antigen at 36 Kd and nonreduced antigen at 71 Kd (Fig. 14). Although all Mabs

TABLE 3. Oocyst output in anti-*Eimeria tenella*, gamont-specific monoclonal antibody (Mab)-treated and sham Mab-treated primary chicken kidney cell (PCKC) monolayers infected with *E. tenella*.

Mab[a]	µg/ml[b]	Count[c]	Oocysts[d]	$SE_{\bar{x}}$	p[e]
GG3	30	7	122.9	18.6	0.564
Control	30	7	101.9	30.1	
GG3	5	6	135.8	39.5	0.456
Control	5	7	100.3	26.1	
HF1	30	6	327.5	48.3	0.396
Control	30	6	391.3	53.4	
HF1	5	7	328.4	30.9	0.581
Control	5	7	357.4	40.8	
EA7	30	8	116.5	13.2	0.047[f]
Control	30	6	164.3	25.0	
EA7	5	7	341.3	25.5	0.034[f]
Control	5	6	239.8	45.8	
FD7	30	9	126.1	11.1	0.080
Control	30	4	162.8	28.9	
FD7	5	10	166.4	20.0	0.469
Control	5	5	163.8	23.6	
GE7	30	11	255.5	20.3	0.190
Control	30	6	222.8	32.1	
GE7	5	2	425.0	10	1.121
Control	5	8	347.5	29.3	
EG3	30	5	330.6	56.8	0.015[f]
Control	30	7	212.0	30.8	
EG3	5	7	169.1	29.9	0.007[f]
Control	5	6	364.7	64.2	
EA12	30	12	67.8	21.8	0.399
Control	30	11	46.0	11.6	
EA12	5	10	50.6	17.2	0.978
Control	5	10	50.0	12.7	

TABLE 3. Continued

Mab[a]	µg/ml[b]	Count[c]	Mean[d]	$SE_{\bar{x}}$	P[e]
FF3	30	4	269.5	66.1	0.928
Control	30	3	278.0	50.9	
FF3	5	4	212.5	47.2	0.327
Control	5	3	306.3	78.9	
HF3	35	5	96	19.5	0.877
Control	35	6	91.2	33.5	
HF3	24	7	75.4	16.9	0.879
Control	24	3	79.7	7.9	
GD9	35	5	83.2	11.9	0.236
Control	35	5	61.8	11.7	
GD9	24	5	134.0	33.5	0.533
Control	24	5	106.9	29.0	
GD9	2.4	7	99.4	25.7	0.734
Control	2.4	8	109.0	12.8	
HD8	35	5	35.4	11.0	0.028[f]
Control	35	5	92.0	18.1	
HD8	24	9	102.4	12.9	0.003[f]
Control	24	9	186.4	20.7	
HD8	2.4	5	181.0	17.3	0.458
Control	2.4	5	152.0	33.0	
HD11	30	10	147.7	20.3	0.799
Control	30	10	159.9	16.0	
HD11	5	10	245.3	26.0	0.393
Control	5	10	203.5	28.6	

[a] Anti-*E. tenella*, gamont-specific Mab added to medium. Sham Mab was of a similar isotype (IgG).

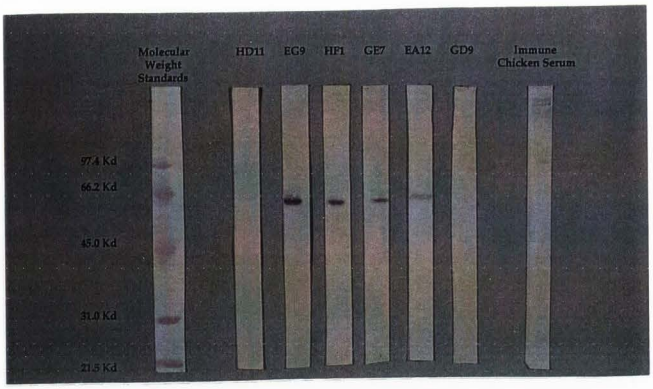
[b] Concentration of Mab added to growth medium on day 3 and 5 postinfection.

[c] Number of PCKC monolayers counted.

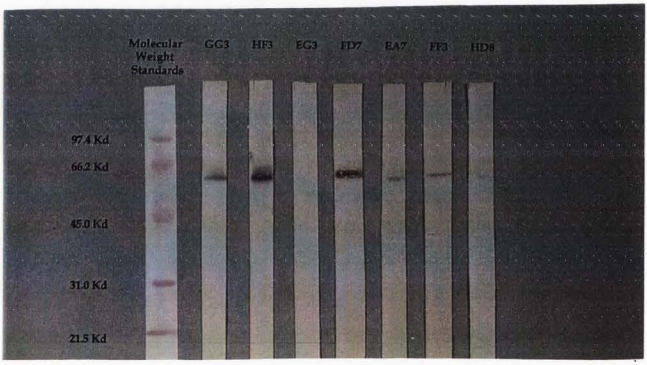
[d] Mean number of *E. tenella* oocysts per cell culture monolayer.

[e] Calculated by using Student's *t* test.

[f] Denotes statistical significance.



A

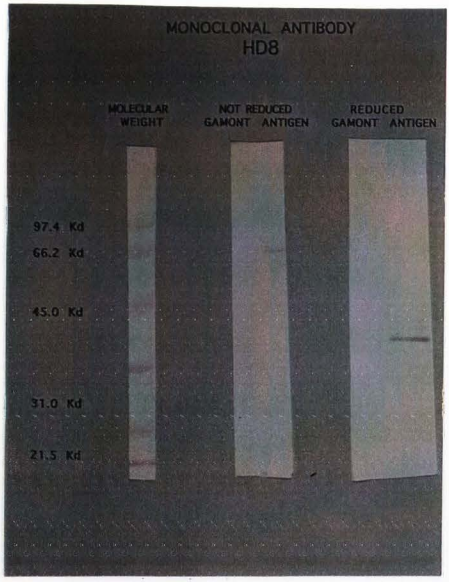


B

FIG. 13. Immunoblots of nonreduced *Eimeria tenella* gamont antigens. Lanes: 1, molecular weight standards with molecular mass (in kilodaltons) noted to left; A. 2-7, individual monoclonal antibodies as listed above individual lanes; 8, immune chicken serum. B. 2-8, individual monclonal antibodies as listed above individual lanes.

recognized nonreduced antigens of the same molecular mass, they did so with varying intensities as determined by the degree of color development in Western blots.

Silver staining of *E. tenella* gamont antigens derived from soluble and membrane-associated fractions had no major protein bands that directly corresponded with target antigens discerned on Western blots. Major protein bands were seen in the soluble fraction at 84, 74, 61, 50, 28, 26, and 22, Kd and in the insoluble fraction at 84, 53, 33, 22, and 20 Kd (Fig. 15). Western blots of nonreduced antigens from soluble and membrane-associated antigens had hybridization only on the membrane-associated lane (Fig. 16). Control blots composed of noninfected cecal antigens and blots lacking primary antibody were all negative when analyzed by Western blot analysis.



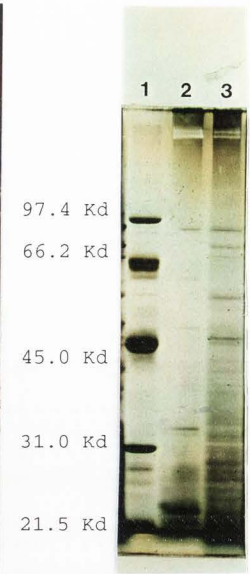


FIG. 14. (Left) Immunoblot of reduced and nonreduced *Eimeria tenella* gamont antigens blotted with monoclonal antibody HD8. Lanes: 1, molecular weight standards with molecular mass (in kilodaltons) noted to left; 2, nonreduced gamont antigen; 3, reduced gamont antigen.

FIG. 15. (Right) Silver stain of *Eimeria tenella* gamont antigens on a 10% SDS-PAGE gel. Lanes: 1, molecular weight standards with molecular mass (in kilodaltons) noted to the left; 2, membrane-associated gamont antigens; 3, soluble gamont antigens.

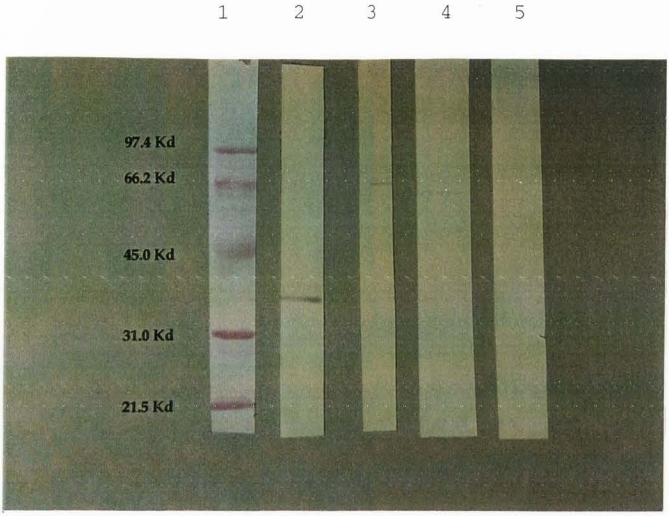


FIG. 16. Immunoblot of *Eimeria tenella* gamont antigens. Lanes: 1, Molecular weight standards with molecular mass (in kilodaltons) noted to the left; 2, reduced *E. tenella* gamont antigens; 3, nonreduced antigens; 4, nonreduced membrane-associated antigens; 5, nonreduced soluble gamont antigens.

DISCUSSION

Adapting *E. tenella* to Cell Culture

Since *E. tenella* was first successfully grown in vitro, researchers have consistently been plagued by low oocyst yields (11-13, 15, 16, 28, 41). This has limited their ability to evaluate drugs in vitro that may effectively inhibit the development of sexual stages in the life cycle of *E. tenella*. Researchers have also been hindered by their inability to study the biochemistry of the *E. tenella* life cycle due to a myriad of problematic variables encountered when studying organisms in their natural hosts (54). Many researchers have spent considerable time and money attempting to develop cell culture lines and methods which more closely simulate in vivo conditions. However, they have been largely unsuccessful in increasing the number of parasites grown in vitro (7, 15-17, 21, 28). We hypothesized that if manipulation of host cell and culture conditions did not dramatically increase the yield of *E. tenella* oocysts, possibly we could develop a cell culture-adapted strain of the parasite to achieve this goal.

Researchers have reported varying numbers of oocysts produced in cell culture using a number of different culture methods, but results have varied greatly (7, 11, 16, 18, 28). Hofmann and Raether (28) reported 13.9-29.9

oocysts per cm^2 when *E. tenella* was grown in cell culture wells. Doran et al. (20) reported between 6-60 oocysts per cm^2 when *E. tenella* was grown in Leighton tubes, depending on the strain of the parasite used. Doran also noted yields of between two and 66.7 oocysts per cm^2, depending on the number of sporozoites used to inoculate tissue cultures (12). In the cell culture-adapted strain of *E. tenella* produced in the present study, the F1 generation yielded an average of 78.5 oocysts per cm^2. By the F5 generation, an average yield of 193 oocysts per cm^2 was achieved. This represents a three- to six-fold increase in the number of *E. tenella* oocysts produced in vitro when compared to results reported by other researchers (11, 28).

Through the development of a cell culture adapted-strain of *E. tenella*, it was our intent to eventually obtain sufficiently large numbers of oocysts so that potential anti-*E. tenella* compounds could routinely be evaluated in vitro for their efficacy against all stages of the parasite, not simply against the asexual stages as is the common practice (36). Eventually, the biochemistry of all stages in the life cycle of *E. tenella* will be elucidated, thus facilitating the development of novel approaches toward controlling avian cecal coccidiosis.

Although the total number of *E. tenella* oocysts produced in vitro varied from one cell culture to the next,

the percent increase in oocysts from the cell culture-adapted strain increased with each generation over the parent strain. Specific factors responsible for the difference in the cell culture-adapted strain of *E. tenella* were not apparent. For example, there were no visual anatomical differences in the cell culture-adapted or parent strain of either the asexual or sexual stages. Additional research is needed to better characterize any ultrastructural differences that may be discerned through electron microscopy.

While supporting data are presently lacking, we believe that the cell culture-adapted strain of *E. tenella* is more pathogenic than the parent strain when used to infect chicks. This empirical observation is based on the increased morbidity noted in chicks administered equal numbers of cell culture-adapted oocysts compared with oocysts of the parent strain. Research addressing the molecular biology of the two parasitic strains may help to resolve this apparent difference in strain virulence.

Observable differences in the behavior of the cell culture-adapted strain of *E. tenella* were apparent from the greater propensity the parasite had for multiple infections in individual PCKC. Primary chicken kidney cells infected with the cell culture-adapted strain were observed to have multiple oocysts growing within them (Fig. 12). This phenomenon of multiple parasites within individual PCKC is

relatively common for the asexual stages of the parent and cell culture-adapted strain. When it occurs, the PCKC nucleus is usually greatly enlarged (Fig. 10). However, multiple oocysts of the parent strain developing within a single PCKC are very rare. Speer (50) reported multiple gamonts of *E. magna* within a single host cell, but noted that many of these degenerated and did not become oocysts. In the present study, it was common to observe two or three oocysts from our cell culture-adapted strain within a single PCKC and occasionally as many as four (Fig. 12). We are unaware of other reports describing multiple oocysts of *E. tenella* residing within individual host cells. Whether the cell culture-adapted strain produces multiple infections because larger numbers of merozoites are released, thus increasing the probability of more than one merozoite penetrating a single PCKC, remains speculative. Another possibility presumes intrinsic differences between strains, thus allowing more than one oocyst to mature within one PCKC.

Some sporozoites of *E. tenella*, immediately upon penetrating PCKC, began undergoing a first round of schizogony, resulting in the release of first-generation merozoites in as few as 48 h (Fig. 4). Other sporozoites apparently reside indefinitely within the host cell and remain protected from the immune system (48). If the chicken's immune system is compromised at some point, these

latent sporozoites may begin to develop, reactivating the infection. This was demonstrated by Long and Millard (34) when they showed that some chickens, apparently immune to *E. tenella,* could be induced to release oocysts after dexamethasone or betamethasone treatment. They also showed that sporozoites of *E. tenella* could survive up to 60 days, even in the presence of the bird's immune system and coccidiostatic drugs. This aspect of *E. tenella*'s life cycle seems to resemble malarial infections in which hypnozoites may remain dormant for years before again causing an active infection (48). Our PCKC system seems to resemble in vivo infections in this aspect. We observed some sporozoites which apparently remained dormant for varying amounts of time before asynchronously developing into trophozoites and first-generation schizonts. How long these sporozoites would remain dormant is unknown since our PCKC system seldom survived beyond 7 days. Nevertheless, intact sporozoites and trophozoites could be seen for up to 7 days within monolayers of PCKC (Fig. 6).

In Vitro Evaluation of Mabs

The results of in vitro evaluation of Mabs for their ability to inhibit the life cycle of *E. tenella* showed the majority demonstrated no significant effect. Lack of inhibition by Mab binding is not surprising in that innumerable different antigenic sites are present on living

organisms. Of the large number of antigens present which may serve a variety of different functions, it is possible that only a very few of these, when bound by Mabs, may impair life cycle completion (64).

More interesting results were obtained with Mabs EA7 and EG3. These two Mabs showed significant inhibition at one concentration and promotion of parasite growth at another concentration. The reasons for this are unclear; however, the standard error of the control PCKC of EA7 and EG3 was significantly higher than the average standard error of the sham Mab treatments. For example, the sham Mab companion to Mab EG3 had a standard error of 64.2 compared with an average standard error of 34.0. This indicates that the Mab PCKC cultures may not have been as homogeneous as others and thereby may not be indicative of the true effect of the Mab on the parasite. Further evaluation of these Mabs must be done to determine their true potential at inhibiting life cycle completion of *E. tenella*.

Monoclonal antibody HD8 showed the most consistently positive results at the highest level of significance. This Mab showed a significant reduction in the number of parasites completing their life cycle at concentrations of 35 µg/ml ($P < 0.05$) and 24 µg/ml ($P < 0.01$), with the lowest concentration of 2.4 µg/ml showing no effect on parasitic development ($P = 0.458$). It remains to be seen if

the target antigen for Mab HD8 serves as a T-cell and/or a B-cell immunogen. In vivo trials are also necessary to determine if immunization with this antigen would produce sufficient immunity to justify the expense of its sequencing and cloning. It would also be valuable to learn the location on the gamont where this Mab binds. This knowledge could aid in determining how Mab HD8 inhibits *E. tenella* growth and development.

Partial Characterization of Mabs

The antigenisity of gamont antigens was lost when they were treated with BME prior to evaluating 12 of 13 Mabs. As a result, it is assumed that these antigens may encompass a disulfide bond, or are very closely associated with the disulfide bonding pattern of the protein. It is important to note that these antigens were boiled in SDS before electrophoresis; the stacking gel and the running gel also contained SDS. The presence of SDS should have linearized and destroyed all tertiary structure of the proteins. Because the Mabs bound to nonreduced but denatured antigens, it appears the targeted antigenic sites on these proteins either contain a disulfide bond or are in some other way greatly influenced by the disulfide bonds of the denatured protein.

Monoclonal antibody HD8 was the only antibody that bound to a reduced and nonreduced gamont antigen (Fig. 15).

The target antigen for Mab HD8 was shown to be membrane-associated, but its exact location, function, and chemical composition are unknown. Analysis by Western blot suggests that the antigen may be a protein consisting of 2 dimers of approximately 36 Kd each, connected by disulfide bonds. Analysis by immunoelectron microscopy is necessary to determine the exact location of the target antigen on the parasitic gamont.

Most Mab binding occurred on Western blots in the 70-73 Kd range (Fig. 13 & 14). However, when SDS-PAGE gels were observed after silver staining of membrane-associated antigens and soluble antigens, no dominant protein bands were observed in this region (Fig. 16). This suggests that target antigens against which the Mabs are directed may not be present in large amounts. Since the gels were only stained with silver and not with other stains, it is possible that there is a stronger protein band present, but its amino acid composition does not lend itself to silver staining. It is also possible that the target antigens are present in large amounts in intact gamonts. If successfully extracted from the gamont membrane, antigens may be readily detectable. The method used to extract membrane antigens employed a mild detergent that may have resulted in incomplete extraction.

The lack of binding in the 70-73 Kd region on immunoblots when probed with immune chicken serum indicates

that these antigens are not very immunogenic. Alternatively, these antigens may not be exposed to the chicken's immune system in a natural infection. However, this latter possibility is quite unlikely.

In studies on *Eimeria maxima* gamonts using immune chicken sera, major immunogenic bands have been demonstrated at 230, 82, and 56 kilodaltons (62). Previous researchers have concluded that these bands may play an important role in immunity to this coccidian (23, 35). From the present data it appears that there may be some major bands shared between *E. maxima* and *E. tenella* in the 82 Kd and 56 Kd range.

The research done with *E. maxima* used immune chicken serum to determine gamont protein banding and the relative immuno-importance of the various bands. In studies where immune chicken serum was used to probe *E. tenella* gamont antigens, a major protein band was observed at approximately 95 Kd; all other major bands observed were >150 Kd (Fig. 13). Additional research is needed to determine if antigens are truly shared between these two *Eimeria* species, or if they simply have major gamont protein bands of approximately the same molecular mass. Sequencing of these proteins from *E. tenella* and *E. maxima* would unequivocally determine if these are identical proteins or if they are simply proteins of similar size.

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