In Situ Enzyme-Linked Immunosorbert Assay To Quantitate In Vitro Development of *Eimeria tenella*

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An in situ enzyme-linked immunosorbert assay was developed to measure in vitro development of *Eimeria tenella*. The assay used a polyclonal, anti-merozoite serum produced by immunization with culture-derived, chromatographically purified merozoites. Although this antiserum cross-reacted with sporozoite-infected cultures (by indirect immunofluorescence and in situ enzyme-linked immunosorbert assay), it clearly distinguished the increase in antigen synthesized throughout intracellular growth. The assay can be used for high-throughput, anticoccidial drug screening, for which it gives quantitative results that are comparable to the published radiometric (³H]uracil incorporation) endpoint.

The demonstration that asexual stages of *Eimeria tenella* could be cultivated in vitro (18) made possible the design of cell culture screens to identify anticoccidial drugs. The first screens described (24) used microscopic observation to score parasite development, a method that was both laborious and qualitative. A more efficient screen endpoint has been described by Schmatz et al. (23), who measured parasite-specific incorporation of [5,6-³H]uracil to quantitate parasite growth. The most labor-intensive step in this radiometric scoring method is the processing of samples for liquid scintillation counting. I describe an alternative scoring method using a colorimetric, in situ enzyme-linked immunosorbert assay (ELISA) which is less laborious and should further improve the efficiency of anticoccidial screening in vitro.

Although ELISAs have been applied to studies of *E. tenella* (3, 9, 16, 17, 21), the methods are not adapted to measuring parasite growth. Instead, they are designed to quantitate the antibody produced in response to infection by using a fixed amount of parasite extract as an antigen. In situ ELISAs have been developed to measure in vitro growth and drug inhibition in virus-infected (1, 2, 11, 20) and protozoan-infected (6, 7, 13) monolayers. These assays detect growth of the infectious agent by measuring the antigen with a fixed amount of antibody specific for the infectious agent. This study was designed to adapt in situ ELISA methods to an *E. tenella* in vitro model.

**MATERIALS AND METHODS**

**Abbreviations.** BSA, Bovine serum albumin; IC₅₀, drug concentration producing 50% inhibition of growth; MDBK, Madin-Darby bovine kidney; OD₄₅₀, optical density at 405 nm; PBS, phosphate-buffered saline.

**In vitro production of merozoites.** MDBK cells (ATCC CCL 22) were grown in continuous culture at 40°C in 3% CO₂ in nutrient mixture F12 (no. 320-1765, Gibco Diagnostics) with 10% (vol/vol) fetal bovine serum (no. 110-1102, J. R. Scientific) and penicillin-streptomycin (no. 600-5140, Gibco). Prior to infection, cells were subcultured into 175-cm² flasks in RPMI 1640 (no. 320-1875, Gibco) with 5% fetal bovine serum (RP5S). When MDBK cells had reached confluence, purified *E. tenella* sporozoites (22) were added at 2.5 × 10⁵/cm² in RP5S. After 24 h of incubation at 40°C to allow invasion, uninvaded sporozoites were removed by agitating the flask, removing the supernatant medium, and washing the monolayer once with RP5S. Fresh RP5S was added, and the cultures were incubated another 40 h, until merozoites were released into the culture medium.

**Antigen and antisera.** The method for merozoite purification was similar to that described for *Plasmodium lophurae* by Hollingdale and Kilejian (10). Briefly, culture supernatants containing released merozoites were collected (1.0 × 10⁶ to 2.5 × 10⁶/cm²) and spun at 450 × g for 10 min to concentrate the merozoites. The pellet, containing merozoites and host cell debris, was suspended in 0.1 M NaCl–0.05 M KCl–20% BSA (no. A7284, Sigma Chemical Co.) and applied to a DE-52 (Whatman, Inc.) column equilibrated in 75 mM Tris–40 mM NaH₂PO₄–86 mM NaCl–100 mM glucose, pH 8.2. The merozoites that flowed through this column were evaluated for purity (removal of host cell debris) by electron microscopy (12). Purified merozoites were suspended in PBS (no. 310-4190, Gibco), disrupted by sonication, emulsified in Freund adjuvant (1:1, PBS-adjuvant), and injected into New Zealand White rabbits (1 × 10⁸ to 5 × 10⁸ merozoites per rabbit). The primary injection was intraperitoneal with complete adjuvant, and subsequent monthly boosts were subcutaneous with incomplete adjuvant. After three boosts, rabbits were bled for serum.

**Indirect immunofluorescence.** MDBGK cells were seeded at 1.3 × 10⁶ cells/cm² in RP5S into six-well tissue culture plates (Falcon 3046, Becton Dickinson Labware) for which each well contained a sterile glass cover slip. After 24 h of incubation at 40°C in a humidified CO₂ incubator, sporozoites were added at 2.7 × 10⁷/cm². Four hours after inoculation, cultures were washed to remove uninvaded sporozoites. Sporozoite removal was achieved by gently flushing the monolayer with culture medium by using a Pasteur pipette and then replenishing the monolayer with fresh medium prior to incubation at 40°C. Some cover slips were fixed at 4 h in −20°C acetone for at least 30 min and then air dried. The remaining cover slips were fixed at 48 h.

Indirect immunofluorescence samples were presoaked in PBS with 0.5% (wt/vol) BSA (PBS-BSA) for 30 min at 40°C, incubated with rabbit anti-merozoite serum (diluted 1:6,400 in PBS-BSA) for 90 min at 40°C, and washed three times in PBS-BSA for 10 min per wash. Samples were then incubated with fluorescein-conjugated sheep anti-rabbit immunoglobulin G (no. 1212-0084, Organon Teknika) diluted 1:100 in PBS-BSA for 90 min at 40°C and washed again three times in PBS-BSA for 10 min each wash. Cover slips of infected
cultures were mounted onto slides and photographed in a Nikon Optiphot microscope with fluorescence attachment.

ELISA. (i) In vitro cultivation. MDBK cells grown for 3 days in RPMI were detached from the monolayer by trypsin treatment, counted in a hemacytometer, and dispensed into 96-well microtiter plates (no. 3595, Costar) at 4 × 10^4 cells per well in 200 μl of RPMI. Cells were not plated in the outer wells of the plate, where severe edge effects (inhibition of parasite development) have been noted (unpublished observations). After 24 h of incubation at 40°C in a humidified 3% CO₂ incubator, the medium was removed, purified sporozoites were added at 4 × 10^4 per well in RPMI 1640 with 2% fetal bovine serum (RP2S), and incubation was continued. The inoculating dose was selected on the basis of experiments showing that when the inoculum was varied from 0.5 × 10^7 to 16 × 10^7 sporozoites per well, 4 × 10^4 per well gave the highest 48-h OD to 4-h OD ratio. For the stage specificity experiments, uninvaded sporozoites were washed away 4 h after inoculation. The wash was achieved by mixing the culture medium three times (by using a Cetus ProGroup liquid-handling device) to suspend sporozoites, discarding this medium, and adding 200 μl of fresh RPMI.

(ii) Fixation. After incubation, the medium from the microtiter plates was emptied by shaking the contents out. To fix the monolayer, the wells were filled with room temperature methanol, incubated for 30 min, emptied, air dried, and stored at room temperature until the ELISA was done.

(iii) Antibody incubations. The basic ELISA buffer consisted of PBS-BSA. After 45 to 60 min of preincubation in PBS-BSA at 40°C, 100 μl of the primary antibody solution (rabbit anti-merozoite serum diluted 1:1,600 in PBS-BSA) was added and incubated for 90 min at 40°C. Unbound primary antibody was removed by washing four times with 500 μl of PBS-BSA by using the automated washing device of the Cetus ProGroup. Secondary antibody (alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G [no. A-8025, Sigma] diluted 1:1,000 in PBS-BSA; 100 μl) was added and incubated for 90 min at 40°C. Unbound secondary antibody was removed as for the primary antibody.

(iv) Substrate. Plates were washed once with substrate buffer (10 mM MgCl₂, 50 mM Na₂CO₃, adjusted to pH 9.8 with HCl), and 200 μl of substrate (1 mg of p-nitrophenyl phosphate [no. 104-105, Sigma] per ml of substrate buffer) was added. Plates were incubated for 60 min at room temperature and then read in a microplate autoreader (model EL310, Biotek) at 405 nm. Readings were blanked against a well containing a fixed monolayer and 200 μl of substrate which had been incubated in ELISA buffer without primary or secondary antibody.

[5,6-³H]Uracil incorporation. Host cell plating and parasite addition were as described for the ELISA. One day after sporozoite addition, [5,6-³H]Uracil (no. NET-368, Dupont, NEN Research Products) was added to 1 μCi/ml. At 24 h after the radiolabel addition, cultures were frozen at −70°C for 1 to 2 h. Thawing resulted in cell lysis and detachment from the plastic. The thawed cultures were harvested with a PHD harvester (model A200, Cambridge Technology), collected onto glass fiber disks (no. 240-1, Cambridge Technology), washed with water, and dried with methanol. After the addition of Aquasol-2 (NEF-952, Dupont, NEN), paper disks were counted in a model 1219 BetaRack liquid scintillation counter (LKB Instrument, Inc.).

Drug screening. Salinomycin (Pfizer Inc.) and clopidol (Dow Chemical Co.) were solubilized in dimethyl sulfoxide (no. D-128 Fisher Scientific Co.) in a concentrated solution and diluted in RPMI such that the dimethyl sulfoxide concentration was <0.25%. Serial dilutions were done in the MDBK cell-containing microtiter plates just prior to sporozoite addition with the Cetus ProGroup. Each drug level was tested in duplicate, and mean values were used to calculate the percent inhibition. For [5,6-³H]Uracil tests, the percent inhibition of growth in the presence of drug was calculated as follows: 100 − [(dpm infected medicated − dpm uninfected)/(dpm infected nonmedicated − dpm uninfected)], where dpm is disintegrations per minute. For ELISAs, the percent inhibition of growth in the presence of drug was calculated as follows: 100 − [(OD infected medicated − OD 4-h infected control)/(OD 48-h infected nonmedicated − OD 4-h infected control)]. IC₅₀, expressed in micrograms per milliliter, was determined by log-linear interpolation between the two drug concentrations giving >50% inhibition and <50% inhibition in a titration.

RESULTS
Merozoite antigen purification. E. tenella merozoites were chosen as the immunogen since (i) they are present in the mature parasites to be assayed by the ELISA and (ii) they can be isolated in an extracellular form. Extracellular merozoites in culture were intermixed with debris from lysed MDBK cells and therefore required purification to avoid raising anti-host cell antibodies. Merozoite purification by anion-exchange chromatography was tested under a variety of ionic conditions. Although increasing ionic strength resulted in higher merozoite yields from the DE-52 column, contamination with host cell debris was greater and the merozoite enrichment was reduced. Under the conditions described in Materials and Methods, merozoite yield from the column has ranged from 30 to 40%. Particulate cellular debris was counted by light microscopy (×400 magnification) before and after column purification; only 3 to 5% of the material applied to the column was present in the effluent. The electron micrographs in Fig. 1 show the culture supernatants prior to DE-52 column purification (Fig. 1A) and after purification (Fig. 1B). Merozoites directly from the culture were surrounded by lysed host cells and cellular debris. After column purification, host cell debris could be detected only after extensive scanning of the thin sections; the predominant image was that of pure merozoites.

Immunofluorescent localization of antibody binding. To assist in the interpretation of ELISA results, anti-merozoite antibody binding to infected cultures was visualized by indirect immunofluorescence (Fig. 2). Sporozoites (Fig. 2A) showed distinct fluorescent staining, while the fluorescence associated with mature schizonts (Fig. 2B) was equally intense but distributed over a greater area. Faint staining was also observed over MDBK cells.

Stage specificity of antibody binding. The amount of antigen (OD₅₀) in infected cultures at various stages of development was evaluated by ELISA. Figure 3 shows a representative experiment. Although there was little antibody binding to uninfected cultures (in most experiments, the OD₅₀ was less than 0.100), a significant amount of antigen was detected even 4 h after inoculation with sporozoites. In infected cultures, antigen did not increase further until 24 h after inoculation. This profile and the immunofluorescence results indicate that the late merozoite serum cross-reacted with sporozoite antigens (4 h). Antigen increased between 24 and 48 h, during the period of late schizogony and segmentation. Beyond 48 h, antigen decreased, possibly because released merozoites were lost when the culture medium was removed prior to fixation.
Drug screening. Salinomycin and clopidol were tested in six-point titrations with both the [5,6-\(^{3}\)H]uracil and ELISA endpoints. IC\(_{50}\)s determined with the ELISA were comparable to those from the [5,6-\(^{3}\)H]uracil endpoint (Table 1). Table 2 shows a sample of the raw data used to generate the IC\(_{50}\)s shown in Table 1 (in this case, results from experiment 2). Overall, several general features were noted in comparing data from the two assays. First, more within-test, plate-to-plate variability was observed in infected, nonmedicated cultures with the ELISA than with the [\(^{3}\)H]uracil endpoint. As attempts to find the source of this variability were unsuccessful, within-plate infected controls were always used in evaluating drug effects by ELISA. Another difference between the two assays could be seen at highly inhibitory drug levels. In most experiments using the [\(^{3}\)H] uracil endpoint, uracil incorporation at high drug levels was equivalent to that in uninfected cultures. In the ELISA endpoint, the OD\(_{405}\) at high drug levels was not as low as in uninfected control cultures. The minimum OD\(_{405}\) was equivalent to a 4-h infected control. This phenomenon is probably due to the fact that although these drugs inhibit intracellular development, they do not inhibit sporozoite invasion in vitro. Therefore, the ELISA was able to detect antigens of the arrested sporozoites in the drug-treated cultures.

**DISCUSSION**

The in situ ELISA described here has several advantages over [\(^{3}\)H]uracil incorporation (23) as an endpoint for in vitro, *E. tenella* drug screening. Although the radiometric endpoint can be automated to handle large numbers of samples, culture harvesting is tedious and liquid scintillation counting is typically carried out only one sample at a time. The ELISA maintains samples in a 96-well format that can be processed and read one plate at a time. The colorimetric ELISA eliminates the expense, hazard, and disposal of radioactive materials necessary for the radiometric endpoint. In addition, the ELISA endpoint does not require any perturbation of the drug-treated cultures during incubation; the radiometric endpoint requires [\(^{3}\)H]uracil addition midway through the growth cycle.

As tools in probing the development of *E. tenella* in vitro, the ELISA and [\(^{3}\)H]uracil endpoints differ in the type of information they provide. The accumulation of antigen measured in the ELISA is significant only in the final 24 h of development (Fig. 3). The effects of a drug that acts in the first 24 h can be detected only by measuring the lack of increase in antigen in the second 24 h. Thus, it would not be possible to define at what stage a drug is acting. The [\(^{3}\)H] uracil endpoint, in contrast, can provide more detailed information throughout in vitro development. The stage specificity experiment in Fig. 3 of Schmatz et al. (23) illustrates this feature quite clearly. The accumulation of incorporated uracil increases in a linear fashion from the beginning of infection, and pulses of [\(^{3}\)H]uracil, even in the first 24 h, show that the newly invaded parasite is very metabolically active. Experiments in this laboratory confirm these observations. Despite this difference, both assays are reproducibly sensitive to potent (salinomycin) and weak (clopidol) anticoccidial agents.

The in situ ELISA methods have been developed with a
polyclonal antiserum raised against purified merozoites. In the simplest sense, intracellular development is merely a process by which merozoites are produced; many of the antigens synthesized during schizogony should be present as constituents of the merozoites used to develop the polyclonal antiserum. The stage specificity experiment (Fig. 3) demonstrates that antigens recognized by the anti-merozoite serum increased as merozoites were produced. Since many antigens are common to both merozoites and sporozoites (4, 5, 25; H. D. Danforth, Abstr. 58th Annu. Meet. Am. Soc. Parasitol. 1983, abstr. no. 65, p. 46–47; P. Thammana and R. H. Schenkel, Fed. Proc. 43:1808, 1984), this increase could be accounted for by the expansion of parasite mass as the parasite divides from one sporozoite into many merozoites. The increase could also represent the emergence of new stage-specific antigens. The heterogeneous nature of the polyclonal antibody used here does not make it possible to distinguish between the two possibilities. It is possible that a merozoite-specific monoclonal antibody or a sporozoite-adsorbed polyclonal anti-merozoite serum could be substituted in the assay to decrease detection of sporozoite-specific antigens.

Selection of an appropriate method for preparation of the monolayer appears to be an important factor for success in development of an in situ ELISA. Several methods have been described for in situ ELISAs on viable cells (8, 19), glutaraldehyde-fixed cells (1, 2, 11, 15, 20), Formalin-fixed cells (7), and methanol-fixed cells (6, 13). For each system, factors such as monolayer stability, antigen lability, antigen location (cell surface or intracellular), and antibody-binding specificity may affect the choice of method. In this case, methanol fixation stabilized the monolayer and allowed antibody access to the intracellular parasites. I have found that alterations in the fixation conditions can affect the ability of the monolayer to bind the polyclonal antiserum. For example, the use of cold methanol fixation to improve antigen preservation resulted in a disproportionate increase in antibody binding to 4-h, sporozoite-containing cultures, and the use of glutaraldehyde fixation led to very high nonspecific binding to uninfected cells (unpublished observations).

The assay system described here may have applications beyond evaluation of intracellular development. For example, substitution of a polyclonal or monoclonal anti-spore- or merozoite antibody in the ELISA could make it possible to detect inhibitors of sporozoite invasion in a short-incubation (e.g.,

![Image](http://aac.asm.org/Downloaded-from)

FIG. 2. Light micrographs of *E. tenella*-infected MDBK cultures stained indirectly with anti-merozoite serum. Sporozoite-containing cultures (A) and schizont-containing cultures (B) were incubated with rabbit anti-merozoite serum and then with fluorescein isothiocyanate-conjugated sheep anti-rabbit immunoglobulin G. Sp, Sporozoite; Sc, schizont. Bar = 20 μm.

FIG. 3. Stage specificity of antibody binding in the ELISA. At 0 h, sporozoites were added to MDBK cell monolayers (infected). After 4 h of incubation, infected cultures were synchronized by washing off uninvaded sporozoites, and samples were fixed for ELISA at various times throughout incubation. Each value for uninfected cultures is the mean of 24 replicates; each value for infected cultures is the mean of 12 replicates. Error bars show 1 standard deviation.

TABLE 1. Comparison of [H]uracil and in situ ELISA endpoints for drug evaluation

<table>
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<tr>
<th>Drug</th>
<th>Expt no.</th>
<th>IC₅₀ (μg/ml) determined by:</th>
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</thead>
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<tr>
<td></td>
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<td>[H]uracil incorporation</td>
</tr>
<tr>
<td>Clopidol</td>
<td>1</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.36</td>
</tr>
<tr>
<td>Salinomycin</td>
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</tr>
<tr>
<td></td>
<td>2</td>
<td>&lt;0.00001</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.0003</td>
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</table>

*In three independent experiments, titrations were done with duplicate samples at each drug concentration.
TABLE 2. Raw data from experiment 2 in Table 1

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<th>Treatment</th>
<th>Conc of drug (μg/ml)</th>
<th>OD (10^-6)</th>
<th>% Inhibition</th>
<th>(H)Uracil</th>
<th>dpm</th>
<th>% Inhibition</th>
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<tr>
<td>Uninfected control</td>
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* — Not applicable.

4-h) test. I have also found that after evaluation of drug-induced parasite inhibition in the ELISA, samples can be reused in a colorimetric, host cell toxicity test adapted from the method of Mirabelli et al. (14).

ACKNOWLEDGMENTS

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LITERATURE CITED