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H. D. Danforth · P. C. Augustine · J. R. Barta
M. C. Jenkins

In vitro and in vivo immunolabeling of sporozoites, schizonts, and sexual stages of *Eimeria acervulina* and *E. tenella* by a species- and stage-cross-reactive monoclonal antibody

Abstract A cross-reactive monoclonal antibody (mAb), designated 1205, was used to study redistribution, parasitophorous vacuole (PV) incorporation, and in situ antigen production during the intracellular parasite development of *Eimeria acervulina* and *E. tenella*. Western-blot analysis of sporozoite preparations showed that the mAb recognized antigenic bands at 55 and 80 kDa. Indirect immunofluorescent antibody (IFA) labeling of sporozoites produced an internal dot pattern. Immunogold electron microscopy (IM) showed labeling of dense granules within sporozoites. The IFA pattern changed to a general-internal label in immature schizonts followed by a surface-tip pattern in mature merozoites both in vitro and in vivo. IM of the asexual stages revealed the same labeling pattern for the in vivo development of both species, and labeling of rhoptries was seen. In vitro, the PV membrane together with amorphous material within the PV was labeled by IFA during schizont development for *E. tenella*. No IM labeling of either the PV membrane or material within the PV was observed. Sexual stages seen in vivo for both species had the general-internal IFA pattern.

Introduction

The characterization and localization of antigens of *Eimeria* spp. by monoclonal antibodies (mAb) has focused predominately on surface antigens of sporozoites and merozoites or specific organelles such as sporozoite refractile bodies (Danforth 1989). Although some work

has been done on stage-cross-reactive mAb by use of indirect immunofluorescent antibodies (IFA) and Western-blot analysis (Danforth and McAndrew 1987), the migration, redistribution, and parasitophorous vacuole incorporation of parasite stage-cross-reactive antigens during in vivo or in vitro development have not been investigated. In this study, we used a cross-reactive mAb (designated 1205) found to label sporozoite dense granules to determine the fate of antigen during the in vitro development of *E. tenella* and the in vivo development of *E. acervulina* and *E. tenella*.

Materials and methods

Sporozoites of *Eimeria acervulina* and *E. tenella* (Beltsville strains 84 and 80, respectively) were excysted as described by Doron and Vetterling (1967) and either washed in phosphate-buffered saline (PBS, pH 7.4) or further cleaned by use of a Leucopak filter technique (Bontemps and Yvore 1974).

The mAb used in this study was produced as previously described (Danforth 1982; Danforth and Augustine 1983). BALB/cByJ mic (Jackson Laboratory, Bar Harbor, Me.) were immunized with three injections of 2×10^5 sporozoites of *E. acervulina* via the tail vein at 2-week intervals followed by a fourth injection 1 week later. At 3 days after the last injection, spleens were removed and the disrupted splenic lymphocytes were fused with P3-X63-Ag8 mouse myeloma cells. Hybridoma cell lines grown in HAT selective medium were screened for secretion of antibody on air-dried sporozoites by IFA testing (Danforth 1982). Selected antibody-positive cell lines were cloned by a serial limited-dilution technique and the supernatants were stored at –20° C until used. One of these clones (designated 1205) was used in this study.

Western-blot analysis of *E. tenella* sporozoite preparations with mAb 1205 was performed as described by Danforth and McAndrew (1987). Approximately 2×10^7 Leucopak-filter-cleaned sporozoites were disrupted with glass beads (100 µm in diameter) by vortexing for 4 min in sample buffer (Laemmli 1970). This material was centrifuged at 13,000 g and soluble proteins were separated under reducing conditions by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12.5% separating gel). Proteins were electrophoretically transferred (100 mA, 4 h) to Immobilon-P (Millipore Corporation, Bedford, Mass.) using the procedure of Towbin et al. (1979). Strips of the Immobilon-P were blocked in 5% powdered milk dissolved in PBS for 10 min and exposed to undiluted 1205 cell-culture supernatant overnight at a

H.D. Danforth (✉) · P.C. Augustine · M.C. Jenkins
United States Department of Agriculture,
Agriculture Research Service,
Livestock and Poultry Science Institute,
Animal Parasitology Unit,
Beltsville, MD 20705-2350, USA

J.R. Barta
Department of Pathology, Ontario Veterinary College,
University of Guelph,
Guelph, Ontario N1G 2W1, Canada

room temperature (RT) of 21° C. Strips were then rinsed in PBS with 0.05% (v/v) Tween-20 and exposed to a 1:500 dilution in antibody buffer [20 mM sodium phosphate (pH 7.4), 0.5 M NaCl, 0.05% Tween-20 (v/v), and 1% bovine serum albumin (v/v)] of affinity-purified rabbit anti-mouse IgG (Miles-Yeda, Miles Scientific, Naperville, Ill.) for 2 h at RT. Following two mouse washes in PBS/Tween-20, strips were incubated at RT for 2 h with affinity-purified goat anti-rabbit horseradish peroxidase conjugate (Miles-Yeda, Miles Scientific), washed in PBS/Tween-20, and then developed in 4-choloro-1 naphthal and 0.05% H_2O_2. The rate of migration (M_r) was determined by comparison with molecular-weight standards (Bio-Rad, Inc., Richmond, Calif.).

Primary chicken kidney (PCK) cells used in the in vitro studies were established as described by Doran (1971) and grown in Linbro plates on glass coverslips in Dulbecco's minimal essential medium (DMEM) with 10% fetal bovine serum. Coverslips were inoculated with approximately 1.25×10^5 E. tenella sporozoites and, after incubation at 40° C in 10% CO_2, were fixed for 2 min in chilled 100% methanol at 2, 6, 24, 48, and 72 h postinoculation (p.i.). The coverslips were stored in PBS at 4° C until used.

Parasites used for the in vivo studies were obtained by inoculating 2-week-old male Sexsal chickens with 1×10^5 or 1×10^6 oocysts of E. acervulina or E. tenella, respectively, and removing an area of the duodenal loop or the cecal neck at 24-h intervals. Tissues were fixed in Carnoy's fixative, embedded in paraffin, sectioned, and mounted on glass sides as previously described (Augustine and Danforth 1986). Sections were deparaffinized in xylene and rehydrated in PBS before IFA testing.

Methanol-fixed PCK cells and rehydrated tissue sections were exposed to undiluted 1205 supernatant and stained for IFA as described previously (Danforth 1983; Augustine and Danforth 1986).

Immunogold electron microscopy (IM) was performed by fixing sporozoites (5 min) or infected chicken tissue (1 h) in 0.5% glutaraldehyde and 3% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.3). Specimens were dehydrated in a graded series of ethanols (25%–70%, v/v) and embedded in LR White resin (Electron Microscopy Sciences, Fort Washington, Pa). Sections were collected on 200-mesh Formvar-coated nickel grids and immunogold-stained. Sections were floated on a drop of PBS (without Ca^{2+} or Mg^{2+}) containing 0.1 M glycine and 1% (w/v) bovine serum albumin for 10 min to block nonspecific binding. After three 5-min washes in PBS with 0.01% fetal bovine serum (wash solution), grids were floated on the undiluted 1205 supernatant for 2 h at RT. Sections were washed three times for 5 min each in wash solution and floated on a secondary antibody (goat anti-mouse IgG conjugated to 10-nm colloidal gold; E.Y. Laboratories, San Mateo, Calif.) diluted 1:50 in wash solution for 2 h. The grids wee again washed in wash solution for 2 h. The grids were again washed in wash solution and rinsed in distilled water. Sections were stained for 10 min in saturated aqueous uranyl acetate and viewed on a Philips 201C electron microscope.

Results

Western-blot analysis of the mAb1205 binding to sporozoite preparations of Eimeria tenella showed that the mAb recognized a prominant antigenic band at 55 kDa (Fig. 1). A lesser antigenic band was present at 80 kDa.

Air-dried sporozoites of E. tenella and E. acervulina labeled with mAb 1205 displayed an internal-dot IFA pattern (Fig. 2). When the same parasites were viewed by electron microscopy, immunogold labeling of dense granules was seen (Fig. 6).

In PCK cells at 2–6 h p.i., IFA labeling of intracellular E. tenella sporozoites had changed to a general-inter-

Fig. 1 Immunoblot of Immobilon-P transfers of Eimeria tenella sporozoite material separated by SDS-PAGE. mAb 1205 reacts with two bands of approximately 55 and 80 kDa. Standards: phosphorylase b (92.500), bovine serum albumin (66.200), ovalbumin (45.000), carbonic anhydrase (31.000), soybean trypsin inhibitor (21.500), and lysozyme (14.400; Bio-Rad)
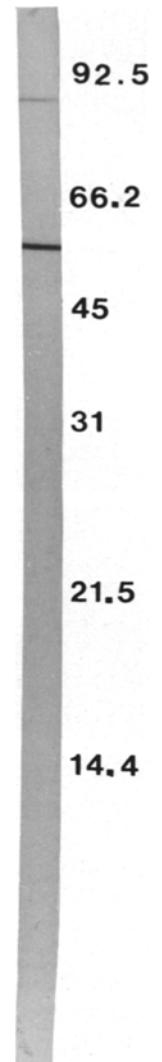
nal type of label (Fig. 3). The same type of IFA label was seen with developing schizonts of E. tenella at 24–36 h p.i. (Fig. 4). Labeling of the parasitophorous vacuole (PV) membrane could be seen in the PCK cells at 24–48 h p.i., and at 24–36 h p.i., amorphous material within the vacoule was also observed (Fig. 4). At 48 h p.i., merozoites could be discerned within the mature first-generation schizonts (Fig. 5).

The same type of general-internal IFA labeling occurred in vivo with the 48 h p.i. development of E. acervulina and E. tenella (Fig. 7). Uniform immunogold labeling was observed with trophozoites of both species at 48 h p.i. /(Fig. 8). No discernable IFA or IM labeling of the PV contents or membrane was seen with the asexual or sexual development of either species at 48–144 h p.i. (Figs. 8–13).

At 72 h p.i., a further change of both the IFA and the IM labeling of mature merozoites was seen in vitro and in vivo (Figs. 9, 10). First-generation in vitro merozoites of E. tenella demonstrated a surface-tip type of IFA pattern (Fig. 9). IM of in vivo mature schizonts of E. acervulina seen at 72 h p.i. showed a similar type of label on
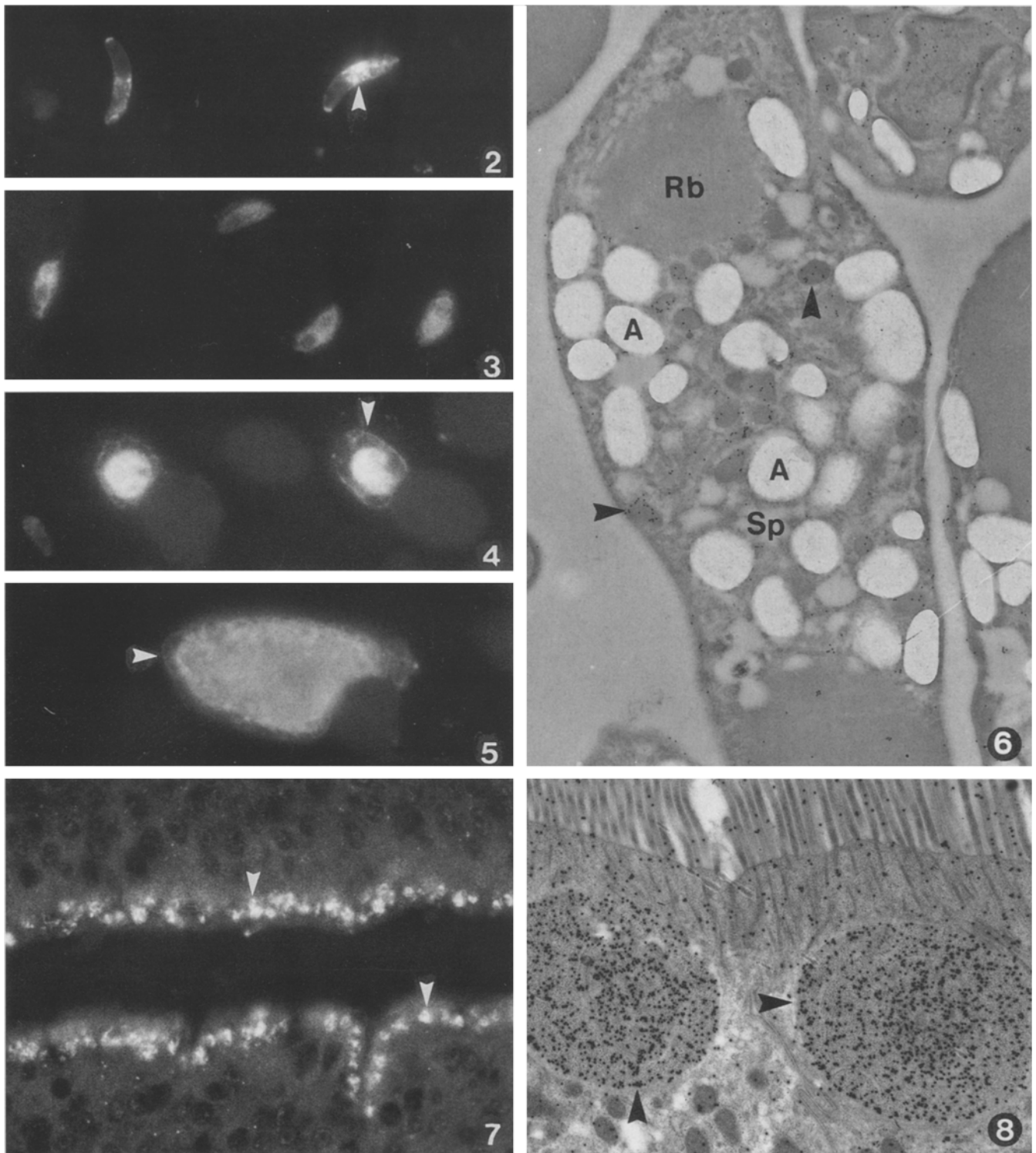
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Fig. 2 IFA micrograph of an air-dried sporozoite of *E. tenella* exposed to mAb 1205. Note the internal-dot IFA pattern (*arrowhead*). × 1,500. **Fig. 3** IFA micrograph of an intracellular sporozoite of *E. tenella* at 6 h p.i. in primary chicken kidney (PCK)-cell culture exposed to mAb 1205. × 1,500. **Fig. 4** IFA micrograph of an *E. tenella* schizont at 30 h p.i. in PCK-cell culture exposed to mAb 1205. Note the labeling of the parasitophorous vacuole (PV, *arrowhead*) and of amorphous material within the PV. × 1,500. **Fig. 5** IFA micrograph of an *E. tenella* schizont at 48 h p.i. in PCK-cell culture exposed to mAb 1205. Note the labeling

of the PV (*arrowhead*). × 1,500. **Fig. 6** IM of an *E. tenella* sporozoite showing gold labeling of dense granules (*arrowheads*). (*A* amylopectin, *Rb* refractile body, *Sp* sporozoites) × 20,000. **Fig. 7** IFA micrograph of chicken intestinal tissue at 48 h p.i. with *E. acervulina* oocysts. Note the IFA-labeled trophozoites and early schizonts (*arrowheads*) in epithelial cells. × 500. **Fig. 8** IM of chicken intestinal tissue at 48 h p.i. with *E. acervulina* oocysts. Note the intense, uniform gold labeling of trophozoites beneath the brush border (*arrowheads*). × 14,000
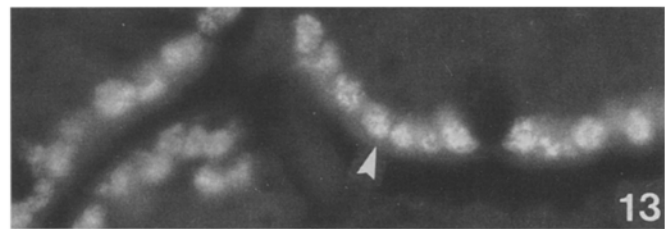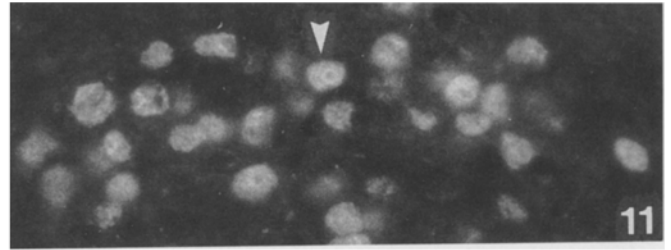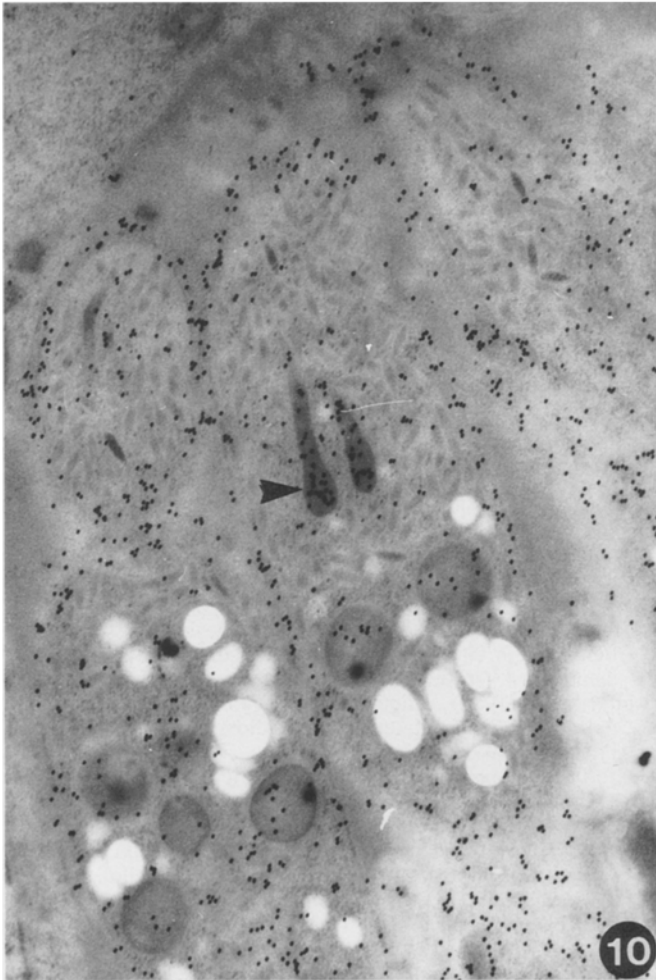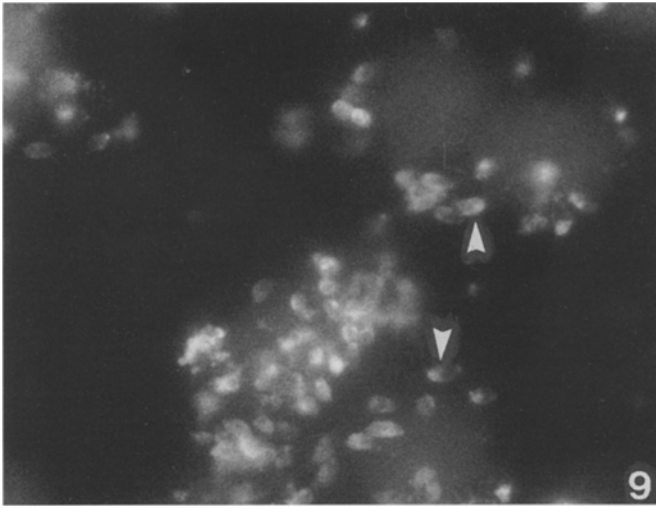
Fig. 9 IFA micrograph of mature extracellular merozoites (*arrowheads*) of *E. tenella* in PCK-cell cultures at 72 h p.i. Note the IFA surface-tip pattern. × 1,500. **Fig. 10** IM of chicken intestinal tissue at 72 h p.i. with *E. acervulina* oocysts. Note that gold labeling has occurred predominantly on the surface of the merozoites within the schizont. Labeling is also visible on the rhoptry structure (*arrowhead*). × 20,000. **Fig. 11** IFA micrograph of chicken cecal tissue at 96 h p.i. with *E. tenella* oocysts. Note the specific labeling of asexual schizont stages (*arrowhead*). × 500. **Fig. 12** IM of chicken intestinal tissue at 96 h p.i. with *E. acervulina* oocysts. Note the uniform gold labeling of a developing schizont stage and the abscence of either material in the PV or the PV membrane. × 14,000. **Fig. 13** IFA micrograph of chicken intestinal tissue at 144 h p.i. with *E. acervulina* oocysts. Note the labeled sexual stages (*arrowhead*). × 500

the surface of the parasites and within the rhoptries (Fig. 10).

The general-internal IFA and IM pattern returned with the early development of the succeeding asexual generations of both species (Figs. 11, 12). As these stages matured, labeling was again seen at the surface of mature merozoites. Sexual stages of both species showed a repeat of the general-internal labeling (Fig. 13).

Discussion

Western blot of mAb 1205 revealed two antigenic bands similar to those reported by other investigators for *Eimeria* species (see reviews by Danforth 1989; Danforth et al. 1993). A comparison between these different antigens within these studies is difficult because of the variability in the percentage of the SDS gels used under both reduced and nonreduced conditions and due to the differences in the types of antibodies (i.e., serum and hybridoma) utilized for Western-blot analysis. All that can be stated is that no correlation between an internal-dot IFA pattern and the 55- and 80-kDa antigens has been reported.

Conformation of the internal-dot IFA pattern was seen with the IM labeling of the dense granules within sporozoites. Very little is known about dense granules of *Eimeria*, but a similar type of immunogold dense-granule labeling has been reported with mAb developed against *Sarcocystis muris* microneme fractions and circulating *Toxoplasma gondii* antigens present in the plasma of infected mice (Entzeroth et al. 1991; Linder et al. 1992). Whether the antigens in dense granules of *Eimeria* sporozoites are involved in penetration of the host cell or are incorporated into the host cell membrane as suggested for other coccidial genea (see reviews by Dubremetz 1990, 1993) requires further investigation. Cross-reactivity studies with mAb 1205 have shown that it does not IFA-label *T. gondii* tachyzoites (unpublished data).

The IFA labeling of the PV membrane and of material within the PV by mAb 1205 at 24 and 48 h p.i. in PCK cells would indicate that there is some antigen incorporation. However, no corroborating IFA or IM labeling of the PV in vivo was demonstrated in this study, which may indicate that the PV labeling is an anomaly of in vitro development. It is also possible that the tissue-fixation techniques used for the in vivo IFA and IM studies had either cross-linkd or destroyed the antigenic epitopes recognized by mAb 1205.

The change of IFA pattern seen with in vivo and/or in vitro studies during the development of *E. acervulina* and *E. tenella* is unique. At least three distinct patterns were seen: first, the internal-dot pattern in sporozoites, followed by a geneal-internal pattern during each generation of asexual development, to eventually a surface-tip type of pattern with mature merozites, with rhoptries also being labeled. A reversion back to a general-internal pattern was seen with the sexual stages. This type of pattern change occurred uniformaly with both species.

Diffusion or migration of antigen during development has previously been reported with mAb that labeled refractile-body material within developing first-generation schizonts of *E. tenella* grown in vitro (Danforth and Augustine 1989). The refractile-body material diffused out into developing first-generation schizonts and then reconcentrated into small dots of material in the mature merozoites. In contrast to the present findings, no further labeling of antigen was seen with continued asexual and sexual stage development, but this type of migration was necessary for schizont development to occur.

Exactly what role the antigens recognized by mAb 1205 have in the life cycle of the parasite is not known. Use of this mAb in the present study demonstrated not only that redistribution and in vitro PV incorporation of parasite antigens could be followed during intracellular development but also that in situ production of antigens could be monitored. The stage- and species-cross-reactivity of these antigens, together with their eventual incorporation into or onto the surface of the merozoites, warrants the further evaluation of their immunopotential.

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