# Molecular Interactions of Cultured Turkey Kidney Cells with Specific Antigens of *Eimeria adenoeides* Sporozoites (42885)

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*Abstract.* **Earlier studies suggested that specific communication between the parasite and the host cell may play a role in cellular invasion by sporozoites of species of avian Eimeria. In this study, quantification of cellular invasion and modified Western blot analysis were used to explore the possibility that parasite receptors for interaction with the host cell might be involved in the sporozoite-host cell communication. Invasion in cultured cells treated with a homogenate of *Eimeria adenoeides* sporozoites was approximately 50% lower than that in untreated cultures. When the sporozoite homogenate was solubilized in sodium dodecyl sulfate and electrophoretically separated, components of the cultured host cells bound consistently to sporozoite bands having $M_r$ of 23 and 40 kDa. Biotinylation of intact sporozoites revealed at least 14 biotin-labeled bands, including bands at 23 and 40 kDa, that were considered to be surface molecules. If the sporozoites were incubated in trypsin after they were biotinylated, only two biotinylated bands at 18 and 23 kDa remained; the 40-kDa biotinylated band was not detected. Despite the removal of the majority of the surface molecules, the cell homogenate still bound to the trypsin-treated sporozoites; the intensity of the label was similar to that resulting from the binding of cell homogenate to untreated sporozoites. The data show specific interactions between 23- and 40-kDa sporozoite bands and host cell components, and provide evidence that the 23-kDa molecule may be located on the sporozoite surface and the 40-kDa molecule located intracellularly.** [P.S.E.B.M. 1989, Vol 191]

In previous studies, certain monoclonal antibodies (CMcAb) produced against cultured primary turkey kidney (PTK) cells were shown to significantly inhibit cellular invasion by sporozoites of *Eimeria tenella* (1). It was proposed that the PTK cell components which were recognized by the CMcAb acted as or formed part of a host cell receptor molecule that may function in the invasion process. The concept of receptor-mediated invasion of cells by protozoan parasites is not a new one. Duffy blood group determinants (2) and sialoglycoproteins (3) are examples of erythrocyte surface molecules that have been shown to function as receptors for merozoites of Plasmodium species. Putative receptor molecules on the surface of mouse fibroblasts have also been identified for attachment of *Trypanosoma cruzi* prior to invasion (Cheryl D. Davis, personal communication). In addition to receptors on host cells, reciprocal receptors on parasite surfaces, for example, *Plasmodium falciparum* merozoites (3), have also been characterized. Specific molecules involved in the interaction of Eimeria sporozoites with host cells have not been characterized, although the site specificity that the sporozoites exhibit in nature (4) and several *in vitro* studies (5–7) suggest that they may exist. This present study was undertaken to determine if there are specific molecules of *Eimeria adenoeides* sporozoites that function as receptors for components of *in vitro*-cultured PTK cells, to establish whether these molecules are located on or within the sporozoites, and to discuss the possibility of their involvement in cellular invasion.

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## Materials and Methods

**Cell Cultures.** PTK cells were prepared as reported earlier (1). Cultures were grown in 24-well tissue culture plates on glass coverslips (15 $mm^2$) seeded with $1 \times 10^4$ cell clumps for invasion studies, or in 75-$cm^2$ tissue culture flasks seeded with 30 ml of medium containing $1.5 \times 10^5$ cell clumps/ml for cell homogenate, membrane isolation, and Western blot procedures. The medium was Dulbecco's minimal essential medium (DMEM) containing 10% fetal bovine serum. All cul-

tures were incubated in 10% $CO_2$ and 90% air atmosphere.

**PTK Homogenate.** Intact PTK cell cultures were washed six times with phosphate-buffered saline (PBS), scraped into 5 ml of 62 m*M* Tris chloride buffer (pH 6.8), and disrupted by vortexing with glass beads (100 $\mu$m in diameter) for 30 sec. The glass beads were allowed to settle. The supernatant fluid was aspirated, diluted approximately 1:1 with 7 m*M* CHAPSO (Sigma Chemical Co., St. Louis, MO), and vortexed for 30 sec. The protein concentration of the homogenate was determined with the BCA microprotein method (Pierce Chemical Co., Rockford, IL) and was adjusted with 62 mM Tris chloride buffer (pH 6.8, containing 10% fetal bovine serum) to contain 0.5 mg of protein/ml.

**Quantitation of Invasion.** Cultures were exposed to homogenates of *E. adenoeides* sporozoites containing either 33 or 66 $\mu$g of sporozoite protein/ml in DMEM or a homogenate of cultured cells containing 300 $\mu$g of protein/ml in DMEM. Control cultures were exposed to DMEM without sporozoite or cell protein. After 30 min at room temperature (RT) (22 ± 2°C), the treatment solutions were aspirated and each culture was inoculated with $3.5 \times 10^5$ *E. adenoeides* sporozoites. In additional experiments, sporozoites were incubated in trypsin (800 units/ml in DMEM; Sigma Chemical Co.) for 30 min at 37°C and washed once with DMEM; $3 \times 10^5$ trypsin-treated or untreated sporozoites were inoculated onto untreated cell cultures. All cultures were incubated for 30 min at 41°C, fixed in 10% buffered formalin, and stained with hematoxylin and eosin. Culture confluency (percentage of the coverslip occupied by cells) and the total number of intracellular sporozoites in 20 equally spaced microscopic fields at ×650 were determined (5). Invasion was expressed as the number of intracellular sporozoites/10 $mm^2$ cells at ×650 (5) after 30-min incubation at 41°C. Each experiment was replicated two or three times with six coverslips per group in each replicate. The data from the experiments were subjected to analysis of variance and Duncan's multiple range procedures and were expressed as the mean ± standard error of the mean of the combined replicates.

**Membrane Enrichment.** Cultures of PTK cells (thirteen to eighteen 75-$cm^2$ flasks) were scraped into 10 ml of 25 m*M* Hepes buffer (pH 9.0) containing 300 m*M* sucrose, 10 m*M* iodoacetamide, and 50 $\mu$g/ml each ribonuclease and deoxyribonuclease. Approximately 3 ml of glass beads were added and the cells were disrupted by vortexing for 2 min. The glass beads were allowed to settle; the supernatant was aspirated and centrifuged at 15,000*g* for 30 min at 4°C. The pellet was discarded and the supernatant was centrifuged at 100,000*g* for 90 min at 4°C. The pellet was suspended in 25 m*M* Hepes buffer (pH 7.6) and the protein content was determined using the BCA microprotein assay method.

**Monoclonal and Polyclonal Antibodies.** CMcAb elicited against cultured PTK cells were developed as reported earlier (1). Polyclonal antiserum against the PTK cell membrane preparation was produced by inoculating rabbits subcutaneously in the neck and hind legs once a week for 4 weeks. Each injection contained from 162 to 400 $\mu$g of protein. For the first injection, the antigen was homogenized in Freund's complete adjuvant; the subsequent injections were in Freund's incomplete adjuvant. Blood was drawn 3–4 weeks after the last immunization and the serum was recovered and frozen. The antibody titers were determined using methanol-fixed PTK cell cultures and standard indirect immunofluorescent antibody techniques.

**Biotinylation of Sporozoites.** Surface proteins of intact sporozoites of *E. adenoeides* (viability >98%) were labeled with biotin using the procedure described by Hurley *et al.* (8). Briefly, $1 \times 10^8$ sporozoites were suspended in 0.5 ml of PBS to which 500 $\mu$g of *N*-hydroxysuccinimidobiotin (Sigma Chemical Co.) dissolved in 50 $\mu$l of dimethyl sulfoxide were added. The sporozoites were shaken at RT for 10 min and centrifuged at 400*g* for 2 min. The pelleted sporozoites were washed once with PBS, subjected to vortexing with glass beads for 2 min, and centrifuged at 400*g* for 2 min at RT. The pellet was washed twice with PBS. Additional groups of sporozoites were biotinylated and then incubated for 15 min in 0.05% trypsin (pH 7.7, 800 units/ml) at 37°C before they were disrupted.

**Gel Electrophoresis and Western Blot.** Biotinylated and unbiotinylated sporozoite preparations and biotinylated molecular weight standards (Bio-Rad, Richmond, CA) were suspended in sample buffer containing 2.3% (w/v) sodium dodecyl sulfate (SDS), 10% (v/v) glycerol, and 5% (w/v) dithiothreitol in 62 m*M* Tris chloride buffer (pH 6.8). The molecular weight standards included rabbit muscle phosphorylase b (97,400), bovine serum albumin (66,200), hen egg white ovalbumin (42,699), bovine carbonic anhydrase b (31,000), soybean trypsin inhibitor (21,500), and hen egg white lysozyme (14,400). Each preparation was boiled for 5 min and centrifuged at 400*g* for 2 min at RT. Approximately 10 $\mu$l of the supernatant fluid, containing 4–5 $\mu$g of sporozoite protein, were applied to each well of a 10-well gel (7 × 8 cm, 0.75-mm thick). Laemmli procedures (9) were followed using 11% polyacrylamide gels (33:1 acrylamide:bisacrylamide) and a Tris-glycine-SDS running buffer (pH 8.3). The electrophoretic separation was conducted using a Mighty Small II Vertical Slab Unit (Hoefer Scientific, San Francisco, CA) at 80 V for 3.5 hr at 4°C. Proteins were subsequently transferred from the gel onto nitrocellulose sheets by a modification of the procedure described by Towbin *et al.* (10). The transfer was carried out at 4°C in a Mini Transfer (Hoefer Scientific) at 35-39 V/200 mA for 4 hr with a Tris-glycine-SDS transfer buffer containing 20% methanol (pH 7.9). The nitrocellulose

sheets were exposed to 5% Blotto (pH 7.5) overnight at RT, rinsed twice with distilled water, and cut into strips.

**Detection of Biotinylated Antigens.** Nitrocellulose strips containing the biotinylated standards and biotinylated sporozoite antigens were incubated in peroxidase-conjugated avidin (Cooper Biomedical, West Chester, PA; 1:500 in antibody buffer) for 2 hr at RT. The strips were washed twice with PBS + Tween 20 and exposed to 4-chloro-1-naphthol (Kirkegaard and Perry Laboratories, Gaithersburg, MD). When the desired color intensity was obtained (usually within 20 min), the reaction was terminated by rinsing the strips with distilled water.

**Binding Assays.** Nitrocellulose strips containing parasite antigens were incubated with the PTK cell homogenate for 6 to 7 hr at 41°C; the strips designated as controls were incubated with Tris chloride buffer (pH 6.8) containing 10% fetal bovine serum and approximately 1 m*M* CHAPSO. The strips were then exposed to rabbit anti-PTK membrane serum at a dilution of 1:200 in antibody buffer (10), normal rabbit serum (1:200 in antibody buffer), or to CMcAb G7, E10, and C8 (undiluted hybridoma culture supernatants) for 4 hr at RT. When the rabbit sera were used, the strips were subsequently incubated in peroxidase-conjugated goat anti-rabbit IgG (H + L chain specific, 1:500 in antibody buffer; ICN Immunobiologicals, Lisle, IL). In experiments involving the CMcAb, the strips were treated first with rabbit anti-mouse IgG (H + L chain specific; 1:500 in antibody buffer, ICN) and then with peroxidase-conjugated goat anti-rabbit IgG (H + L chain specific, 1:500 in antibody buffer; ICN Immunobiologicals). The color reaction was initiated with 4-chloro-1-naphthol and terminated as described above. The nitrocellulose strips were washed with PBS + Tween 20 between each step.

## Results

**Invasion.** Cultured cells exposed to either 33 or 66 μg of sporozoite homogenate protein prior to inoculation with viable *E. adenoeides* contained significantly fewer sporozoites than control cultures exposed to DMEM (Table I, Experiments 1–3). In contrast, invasion of cultures treated with up to 300 μg/ml of cultured cell homogenate did not differ significantly from that in untreated controls (Table I, Experiment 4). The treatment caused no significant change in culture confluency or gross morphology, and little debris was observed in the cultures. Sporozoites that were exposed to trypsin before they were inoculated into cultures invaded PTK cells as efficiently as did their untreated counterparts (treated = 7.81 ± 1.41; control = 8.33 ± 1.96).

**Biotinylation of Sporozoite Surface Molecules.** Biotinylation of intact sporozoites of *E. Adenoeides* revealed major surface antigens having $M_r$ of approximately 23, 50, and >100 kDa, and at least 11 other bands with varying amounts of biotin label from approximately 18 to >100 kDa (Fig. 1, Lane a). Viable sporozoites that were incubated overnight at 4°C in DMEM before they were exposed to biotin had the same labeling pattern as those that were biotinylated shortly after excystation (data not shown). When the biotinylated sporozoites were incubated in trypsin before they were disrupted for analysis, only bands at 18 and 23 kDa were still detected (Fig. 1, Lane b). No bands were detected in any control preparations in which sporozoites were not exposed to biotin.

**Binding of PTK Antigens to Sporozoite Antigens.** Rabbit anti-PTK membrane serum and CMcAb C8 were used to detect binding to PTK cell components to *E. adenoeides* sporozoite antigen. These reagents pro-

**Table I.** *Eimeria adenoeides* Sporozoite (Sz) Invasion of Cultured Cells that were Pretreated for 30 min with a Homogenate of Sporozoites of the Same Species (Experiments 1–3) or a Homogenate of Host Cells (Experiment 4)

| Experiment | Treatment[a] | *N*[b] | Culture confluency[c] (%) | Intracellular | |
|---|---|---|---|---|---|
| | | | | Sz/20 fields[c] | Sz/10 mm² cells[c] |
| 1 | 0 | 6 | 28 ± 5 | 38.3 ± 14.4 | 9.62 ± 2.72 |
| | 33 | 6 | 33 ± 5 | 27.7 ± 12.6 | 5.85 ± 2.33* |
| 2 | 0 | 6 | 16 ± 2 | 20.8 ± 8.6 | 8.62 ± 4.52 |
| | 66 | 6 | 18 ± 3 | 10.5 ± 6.1 | 4.04 ± 2.06* |
| 3 | 0 | 6 | 16 ± 2 | 15.8 ± 7.2 | 6.87 ± 2.51 |
| | 33 | 6 | 16 ± 3 | 5.5 ± 2.9* | 2.52 ± 1.09* |
| | 66 | 6 | 15 ± 3 | 6.3 ± 1.6* | 2.93 ± 0.42* |
| 4 | 0 | 6 | 37 ± 3 | 18.0 ± 4.0 | 3.35 ± 0.52 |
| | 300 | 5 | 38 ± 3 | 13.0 ± 3.9 | 2.33 ± 0.53 |

[a] Micrograms of sporozoite or cell protein applied to each coverslip.
[b] Number of coverslips quantified.
[c] Data expressed as mean ± standard error of the mean; means followed by asterisks are significantly different from the control mean of the parameter in that experiment.

duced similar indirect immunofluorescent antibody reactions on methanol-fixed PTK cell cultures (Fig. 2). When the rabbit serum was used as the probe, binding of the PTK homogenate to 23- and 40-kDa sporozoite bands was consistently detected (Figs. 3, Lane a and 4, Lane a) and binding to three bands at approximately 60 kDa was observed infrequently. On blots probed with CMcAb C8, binding to only the 40-kDa sporozoite antigen was evident, and the intensity of the label was far less than that obtained with the anti-PTK serum (Fig. 3, Lane b). No bands were detected on sporozoite antigens exposed to rabbit anti-PTK serum or CMcAb C8 without prior exposure to the PTK homogenate, or when the antigens were exposed to PTK homogenate and then to normal rabbit serum. Similarly, no bands were detected on blots probed with two other CMcAb, E10 and G7.

Biotinylation of the sporozoites before they were disrupted did not appear to inhibit the binding of the cell homogenate to the sporozoite antigens. Figure 4 shows preparations of unbiotinylated (Lane a) and biotinylated (Lane b) sporozoites. Both of the strips were exposed to the cell homogenate and rabbit antimembrane serum, and then developed with peroxidase-conjugated antirabbit IgG. No avidin was included in the procedure. Bands were detected at 23 and 40 kDa in both the biotinylated and unbiotinylated sporozoite

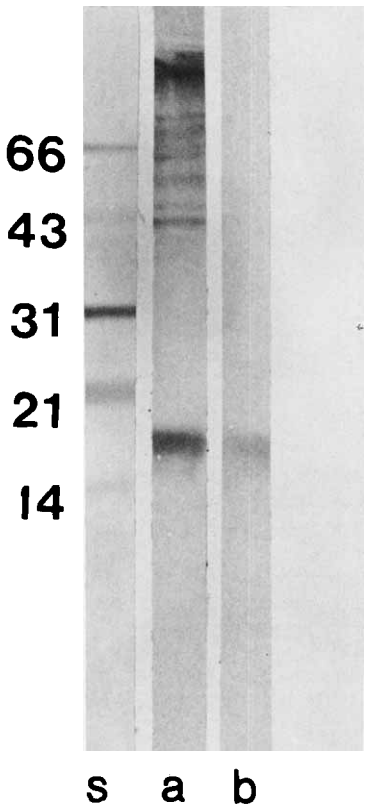


**Figure 1.** Pattern of surface proteins of intact *Eimeria adenoeides* sporozoites that were exposed to *N*-hydroxysuccinimidobiotin, electrophoretically separated, and exposed to peroxidase-conjugated avidin. In Lane a, the sporozoites were solubilized in SDS immediately after biotinylation. In Lane b, the biotinylated sporozoites were incubated in 0.05% trypsin for 15 min at 37°C before they were solubilized in SDS.

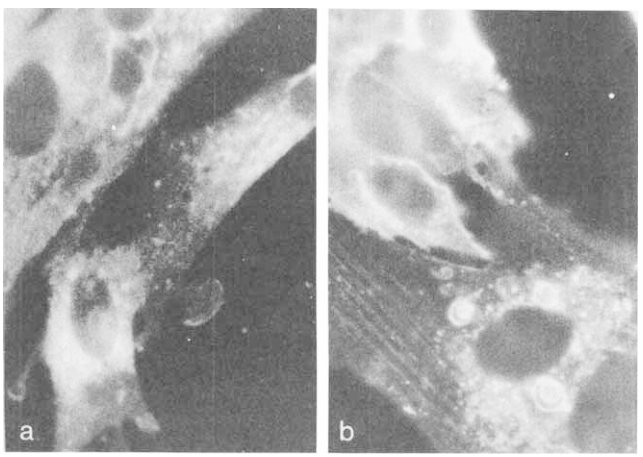


**Figure 2.** Methanol-fixed cell cultures labeled with rabbit anticell membrane serum (a) and monoclonal antibody C8 (b) using indirect fluorescent antibody techniques. These antibodies identified the binding of cell homogenate to specific sporozoite molecules (original magnification ×800).

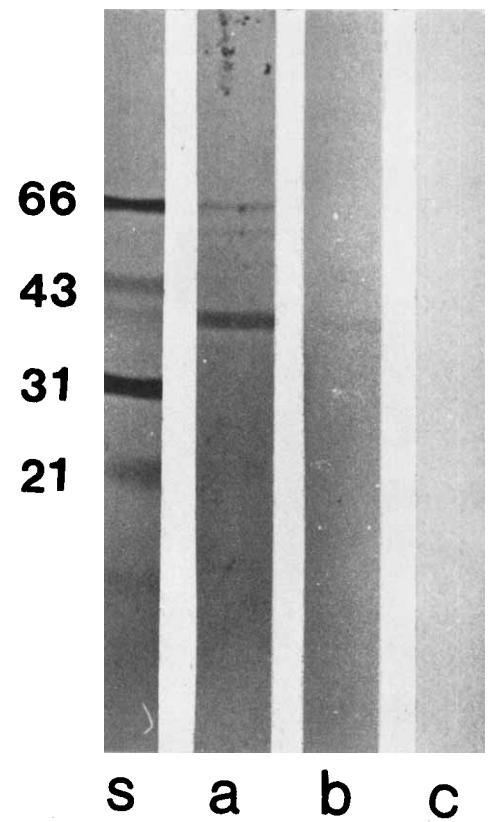


**Figure 3.** Modified Western blot analysis of *E. adenoeides* sporozoites showing binding of host cell homogenate to sporozoite antigens. The sporozoite antigens were electrophoretically separated and exposed to cell homogenate and then to rabbit anticell membrane serum (Lane a) or monoclonal antibody C8 (Lane b). Binding of cell homogenate was detected with peroxidase-conjugated antirabbit (Lane a) or antimouse (Lane b) IgG. Lanes s and c contain biotinylated standards and control strip, respectively.

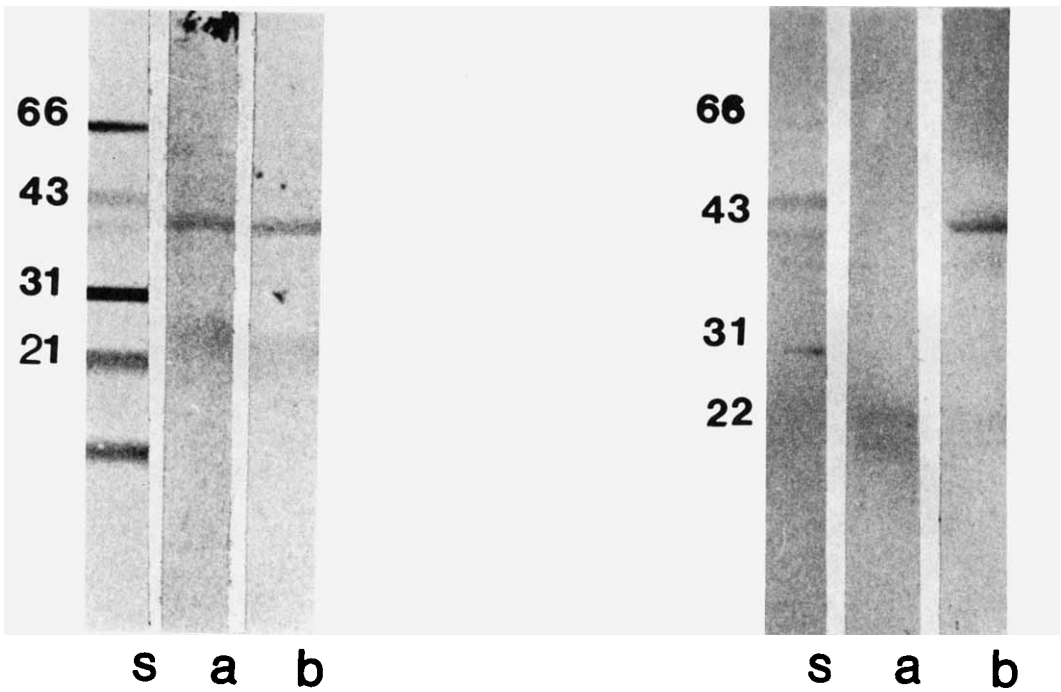


**Figure 4.** Modified Western blot analysis of *E. adenoeides* sporozoites showing binding of host cell homogenate to sporozoite antigens. In Lane a, the sporozoites were not biotinylated. In Lane b, the sporozoites were biotinylated before they were disrupted for the binding assay. Both antigen preparations were exposed to cell homogenate, rabbit anticell membrane serum, and peroxidase-conjugated antirabbit IgG. Lane s contains biotinylated standards that were developed with peroxidase-conjugated avidin.




**Figure 5.** Modified Western blot analysis of *E. adenoeides* sporozoites showing binding of host cell homogenate to trypsin-treated sporozoites. The sporozoites in both lanes were biotinylated and then exposed to trypsin before disruption for analysis. In Lane a, the strip was subsequently exposed to peroxidase-conjugated avidin and showed only the trypsin-resistant 18- and 23-kDa bands. In Lane b, the strip was exposed to the cell homogenate, rabbit anticell membrane serum, and peroxidase-conjugated antirabbit IgG. Lane s contains biotinylated standards that were developed with peroxidase-conjugated avidin.

preparations. Similarly, exposure of sporozoites to trypsin prior to disruption did not affect the binding. In both lanes of Figure 5, the sporozoites were biotinylated and then trypsinized before the binding assay. On the strips subsequently exposed to peroxidase-conjugated avidin (Lane a), only the trypsin-resistant 18- and 23-kDa bands were detected. The same antigen preparation exposed sequentially to the cell homogenate, anticell membrane serum, and antirabbit IgG (Lane b) showed binding of cell components to the 23- and 40-kDa bands.

## Discussion

Treatment of cultured PTK cells with a homogenate of disrupted *E. adenoeides* sporozoites before inoculation with viable sporozoites of the same species reduced cellular invasion by approximately 50% as compared with that in the untreated cultures. One explanation for this occurrence is that sporozoite proteins in the homogenate may have bound to PTK cell molecules prior to the introduction of the viable sporozoites, thus limiting the viable sporozoites' interaction with the host cell. The inhibition of invasion produced by the sporozoite homogenate was apparently due to specific parasite-host cell interactions rather than nonspecific adherence, since invasion was not reduced in cultures similarly treated with a homogenate of host cells. The treated cultures did not differ from the untreated cultures in confluency or cell morphology at the light microscope level, suggesting that the decrease in invasion was not due to gross changes in the structure of the cells. That little debris was seen in the treated cultures indicated also that the decrease in invasion was not caused by a general blockade of the cell surface by the sporozoite homogenate.

A modified Western blot analysis of the disrupted *E. adenoeides* sporozoites identified specific bands to which components of the PTK cells bound. Electrophoretic separation and Coomassie blue staining of *E. adenoeides* sporozoites disclosed a large number of polypeptide bands. When this array of bands was transferred to nitrocellulose sheets and exposed to the PTK cell homogenate and then to anti-PTK cell antibodies, only a light band at 23 kDa, a more intensely labeled band at 40 kDa, and, infrequently, three light bands at around 60 kDa were detected. To investigate the location of the 23- and 40-kDa antigens, biotinylation and enzymatic digestion of surface proteins of *E. adenoeides* sporozoites were combined with the binding assay. The antigens at approximately 60 kDa were not examined further for this study.

Biotin-labeling techniques, wherein biotin attaches

to primary amino groups (11, 12), have been used to identify surface antigens of a number of different cell types, including rat erythrocytes (12) and bovine leukocytes (8). Surface proteins of intact sporozoites of *E. adenoeides* were labeled with biotin using the same technique. Biotinylation did not appear to harm the sporozoites because most of them remained refractile during the procedure, an indication that they were still viable. A typical electrophoresis pattern of the biotinylated sporozoites showed at least 14 biotin-labeled bands which were considered to be surface proteins or polypeptides of the sporozoites. In general, the molecular weights of biotin-labeled bands were similar to those of sporozoites of *Eimeria acervulina* and *E. tenella* that were labeled with $^{125}I$ in previous studies (13–15).

Bands of 23 and 40 kDa were biotinylated, suggesting initially that the 23- and 40-kDa antigens that were bound by the PTK cell homogenate might be located on the sporozoite surface. However, when the sporozoites were biotinylated and then incubated in trypsin, which cleaves lysine and arginine residues (16) and has been shown to remove most if not all of the proteins from cell surfaces (8), the number of bands in the electrophoresis pattern was reduced from 14 to 2. Only bands at 18 and 23 kDa remained; the band at 40 kDa was not detected. Even with the decrease in sporozoite surface proteins, the binding of the PTK homogenate to the 23- and 40-kDa antigens was comparable to that of sporozoites that had not been exposed to trypsin. Therefore, if the 40-kDa antigen is proteinaceous, it is probably located intracellularly and became available to bind with PTK cell components only after the sporozoites were disrupted for analysis. However, if the 23-kDa protein that was bound by the PTK cell homogenate was also biotinylated, then that antigen may be a trypsin-resistant sporozoite surface protein.

It is tempting to speculate that the sporozoite molecules that were bound by the PTK homogenate might be involved in cellular invasion. Although conclusive evidence is not available at this time, a reasonable argument can be made for such a role for the 23-kDa antigen. First, sporozoites have been shown to initiate invasion by repeatedly probing the host cell and may eventually attach to the host cell surface (17). The location of the 23-kDa antigen on the sporozite surface would facilitate interaction with the host cell. Second, its resistance to degradation by trypsin would also be an important attribute. Sporozoites are exposed to trypsin in the lumen of the intestine and during *in vitro* excystation, and remain capable of invading cells thereafter (18, 19). Finally, if the 23-kDa antigen is involved in cellular invasion, then perturbation of this molecule would be expected to result in an inhibition of invasion. Augustine and Danforth (20) described five monoclonal antibodies that inhibited sporozoite invasion of PTK cells. Each of the antibodies reacted with the surface of *Eimeria* sporozoites and recognized a sporozoite antigen reported as 24 kDa (21). Murray and Galuska (15) also identified a 23-kDa polypeptide that is associated with the surface of *E. tenella* sporozoites and that is capable singly, or in combination with other antigens, of inducing immune responses in chickens. Relationships among the 23- and 24-kDa antigens described in the above reports and the one identified in the present research must be determined by comparative studies.

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