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Characterization of Rhoptry Proteins of *Eimeria tenella* Sporozoites: Antigenic Diversity of Rhoptry Epitopes within Species of the Genus *Eimeria* and among Three Asexual Generations of a Single Species, *E. tenella*

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Rhoptry organelles from sporozoites of the apicomplexan parasite *Eimeria tenella* contain at least 60 independent polypeptides that can be resolved by two-dimensional gel electrophoresis. Rhoptries from three species of *Eimeria* that infect chickens share very few antibody cross-reactive epitopes, and there is poor conservation of epitopes among three distinct asexual generations of zoites within the developmental life cycle of a single parasite, *E. tenella*.

Rhoptries are electron-dense, membrane-bound, club-shaped organelles located at the apical end of the invasive stages of all apicomplexan parasites. Rhoptry contents are secreted from the apical end of the parasite into the expanding vacuole at the time of host cell invasion (1, 9) and are thought to play a critical role in the invasion process (17). All apicomplexans are obligate intracellular parasites which enter their host cells by a series of steps that include destabilization of the host cell membrane and formation of an intracellular parasitophorous vacuole that surrounds the newly invaded parasite. Although apicomplexans display greatly varying host cell tropisms, the mechanisms by which entry is established are likely to be conserved, and thus it is reasonable to assume that the interactions of rhoptry contents with the host cell will be similar. However, despite the architectural and, presumably, functional conservation of rhoptries, biochemical and molecular analyses of a limited number of proteins contained within them has so far failed to reveal striking homologies or rhoptry-specific signatures among genera, although recently a number of short oligopeptide motifs semiconserved between the Pf83/Pk66 proteins of *Plasmodium* spp. and the RAP-1 proteins of *Babesia* spp. were noted (23). It is likely that extensive characterization of genes coding for rhoptry proteins from each genus will be necessary to determine whether there are conserved sequences that are central to the invasion processes.

Rhoptries have been purified from several apicomplexan parasites and shown to contain a complex mixture of polypeptides (5, 6, 10, 11, 14). On the basis of electrophoretic profiles of total proteins, rhoptries from various genera appear to share a grossly similar organization. For example, they all have clusters of polypeptides that migrate in the 45- to 65-kDa range, and all have a number of high-molecular-weight proteins. Polypeptides of purified rhoptries from *Eimeria tenella* sporozoites (Fig. 1) were separated by two-dimensional gel electrophoresis (16) and resolved into at least 60 polypeptide spots showing a complexity similar to that of rhoptries from *Toxoplasma gondii* tachyzoites (11). However, the overall profiles are distinct, and in particular, the cluster of abundant proteins of around 55 to 65 kDa in *E. tenella* rhoptries are acidic (pI <4.0) whereas those of *T. gondii* (designated ROP-2, ROP-3, and ROP-4) are basic (pI >8.0).

Several rhoptry proteins from various genera are antigenic, containing both B- and T-cell epitopes (4, 11, 20, 22), and some are potential antigens for inclusion in recombinant vaccines (18). Interestingly, there are no reports of serological cross-reactivity among rhoptry antigens from different genera, and it is thought that there is considerable antigenic diversity. Within a genus, interspecies comparisons of total rhoptry proteins have not been reported until now, but for a number of individual proteins, immunological relationships have been examined. Some antigens are well conserved; e.g., the RAP-1 antigens of *Babesia* spp. are present in all species examined (3). Others display species specificity; e.g., epitopes of the high-molecular-weight rhoptry complex of *Plasmodium falciparum* are not detected at all in the simian malarias, whereas related epitopes are detected, but not in the rhoptry, in murine and human species (2, 4).

To address the question of the immunological relationships of rhoptry polypeptides within the genus *Eimeria*, whole organelles were isolated from sporozoites of three species of *Eimeria* that infect chickens (10) and were examined by Western blotting (immunoblotting). Polypeptides of purified rhoptries were probed with three sera raised to rhoptries of *E. tenella* (Fig. 2). The sera, raised by subcutaneous immunization of rabbits, mice, and chickens with gradient-purified rhoptries, recognize different numbers of polypeptides in the homologous *E. tenella* rhoptry sample, with the rabbit serum binding to the most bands. All three sera cross-react poorly with rhoptries from the two other species of *Eimeria*, staining a small number of polypeptide bands. The major cross-species reactive band migrates at between 55 and 58 kDa, which is similar in size to the cross-species RAP-1 antigens of *Babesia* spp. There is no apparent cross-reactivity with a previously described set of antigens of *Eimeria acervulina* merozoites that are recognized by a monoclonal antibody which binds rhoptries (8). The three sera were used to probe blots of purified rhoptries from the sporozoites of five strains of *E. tenella* (one laboratory strain and four independent field isolates), and no differences in binding between the strains were revealed (data not shown). Thus, there appears to be complete immunological conservation of rhoptry epitopes among sporozoites of strains of *E. tenella* but very little conservation of epitopes among sporozo-

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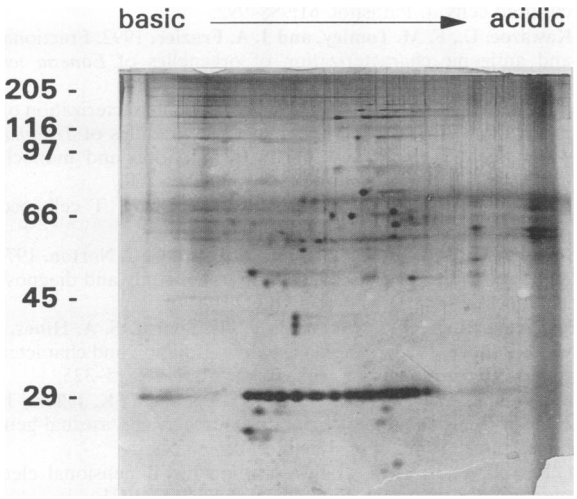

FIG. 1. Polypeptides of gradient-purified rhoptries (from approximately 3×10^7 sporozoites) were separated by two-dimensional gel electrophoresis and stained with silver. Isoelectric focusing was carried out in 0.2% Biolytes 3-10 (Bio-Rad), and marker spots (carbamylated carbonic anhydrase, 29 kDa, pI range of 4.8 to 6.7; Pharmacia) were included on the gel. Molecular weight standards (in thousands) are indicated.

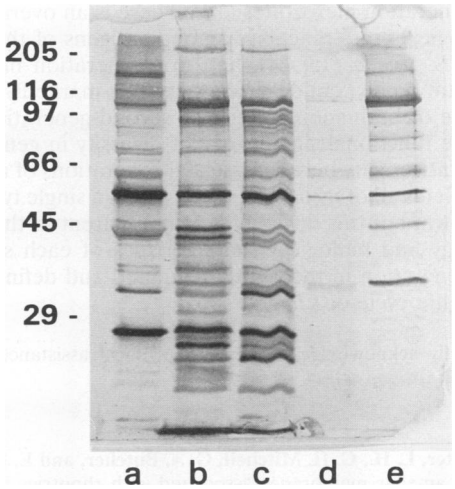

FIG. 3. Polypeptides of purified developmental stages of *E. tenella* were blotted onto nitrocellulose and probed with antiserum against purified rhoptries from sporozoites of *E. tenella* raised in rabbits. Lanes: a, unsporulated oocysts; b, sporulated oocysts; c, sporozoites; d, first-generation merozoites; e, second-generation merozoites. Each track was loaded with 100 μg of total protein, which is equivalent to approximately 5×10^5 oocysts, 5×10^6 sporozoites and second-generation merozoites, and 10^7 first-generation merozoites. Molecular weight standards (in thousands) are indicated.

ite rhoptries of *E. tenella*, *E. acervulina*, and *Eimeria maxima*. Since all three species of parasite invade and develop within epithelial cells of the avian intestine, it might be predicted that the polypeptide composition of their rhoptries would be well conserved. The lack of immunological conservation among rhoptry epitopes is striking and, most interestingly, parallels the exquisite parasite species specificity of the induction of protective immune responses. Immunity to *Eimeria* spp. is mediated primarily through cellular responses which are thought to be induced by the developing intracellular stages (12, 25), but it is not known whether rhoptry antigens are targets for this immunity.

Within a single parasite life cycle, rhoptry diversity among sporozoites and merozoites has been demonstrated in *P. falciparum* by the failure of monoclonal antibodies against merozoite rhoptry proteins to bind to those of sporozoites (4, 19). In contrast, for *T. gondii*, at least two rhoptry proteins are conserved between tachyzoites and bradyzoites (21). The developmental life cycle of *E. tenella* consists of three morphologically distinct asexual replications followed by gametogony and oocyst formation, and all of the intracellular development of the parasite occurs within intestinal epithelial cells of the cecum. To examine both the expression of rhoptry polypeptides during sporulation and the immunological relatedness of rhoptries from different invasive stages of the life cycle, purified preparations of unsporulated oocysts, sporulated oocysts, sporozoites, and first- and second-generation merozoites (10) were examined by Western blotting with rabbit antiserum raised to sporozoite rhoptries (Fig. 3). Many rhoptry-specific epitopes were detected in the unsporulated oocysts, several hours before the rhoptry organelles were assembled. This is consistent with studies on rhoptry biogenesis in *P. falciparum* merozoites which indicate that rhoptry polypeptides are synthesized several hours before they are compartmentalized in the organelle (7). During sporulation, there appears to be some processing of rhoptry-specific polypeptides so that fully sporulated intact oocysts contain essentially the same reactive bands as those contained within purified sporozoites, although there are some subtle differences which may result from the proteolytic processing of rhoptry polypeptides during in vitro excystation of sporozoites, which includes incubation in trypsin (13). First-generation merozoites contain very few polypeptides that react with the anti-sporozoite rhoptry serum, whereas second-generation merozoites have a reactivity profile that more closely resembles that of the sporozoite, although fewer bands are recognized. This pattern of reactivity is in agreement with that found in previous work from this laboratory which indicated that most sporozoite rhoptry epitopes are not recognized by antisera raised to either purified first- or

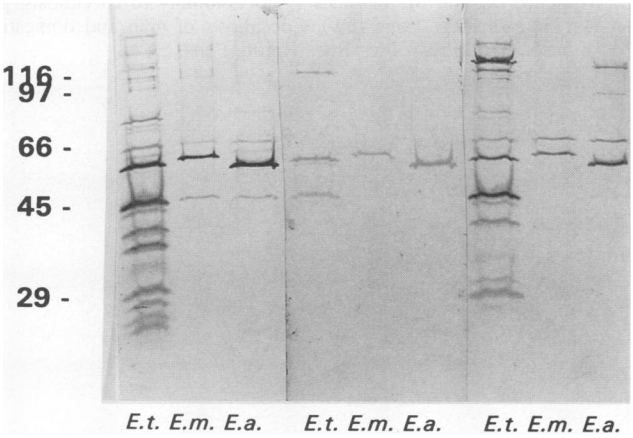

FIG. 2. Polypeptides of gradient-purified rhoptries (from approximately 10^7 sporozoites per track) of *E. tenella* (*E.t.*), *E. maxima* (*E.m.*), and *E. acervulina* (*E.a.*) were blotted onto nitrocellulose and probed with antisera against purified rhoptries from sporozoites of *E. tenella* raised in rabbits (left panel), mice (middle panel), or chickens (right panel). Molecular weight standards (in thousands) are indicated.

second-generation merozoites (10). There is an overall lack of immunological cross-reactivity among antigens of the asexual stages of *E. tenella* (15, 24), and first-generation merozoites display a total polypeptide profile which is markedly different from those of both sporozoites and second-generation merozoites. The functional significance of diversity in gene expression and antigenicity between asexual generations of a parasite that completes all of its development within a single type of cell is not known, but this diversity is also apparent in the distinct morphology and biological characteristics of each successive asexual generation in the tightly regulated and defined developmental life cycle of *Eimeria* spp.

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