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Eimeria tenella: Localization of Cross-reacting Epitopes in Rhoptry, Nucleus, and Host Cell Nucleus during Parasite-host Cell Interactions by a Monoclonal Antibody in PCKC Culture*

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*Dedicated to the 4th anniversary of Prof. Dr. Gerhard Piekarskis death

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ABSTRACT

A cross-reacting mab B1C4 was obtained by fusion of x63-Ag 8.653 plasmacyioma cells with splenocytes. In the immunoblot of sporozoite proteins separated by SDS-PAGE, by mab B1C4 recognized protein bands at 94, 66, 60, 45, and 20 kDa. Antigens recognized by mab B1C4 were localized in rhoptries and nuclei of sporozoites as shown by immunoelectron microscopy, but also in nuclei of uninfected PCK cells as shown by IFAT. Before cell invasion B1C4 labelled granular structures in the apical poles and in the medial cytoplasmic region of sporozoites, stage I merozoites and stage II merozoites. 24 hrs after infection B1C4 labeled a net-like connection between the intracellular sporozoite and the host cell nucleus which was obviously enlarged in infected cells.

INTRODUCTION

According to two-dimensional gel electrophoresis (Tomley 1994), rhoptry organelles from sporozoites of the apicomplexan parasite *Eimeria tenella* contain at least 60 independent polypeptides. In *Plasmodium falciparum* most rhoptry proteins identified by monoclonal antibodies (mabs) also represent potential immunogens for inclusion in recombinant subunit vaccines (Ridley et al. 1990; Perkins 1992). A subunit vaccine would protect the host against the parasite by

induction of a humoral and cellular immune reaction (Lillehoj and Trout 1993), but would lead to autoreactive effects if the components of subunit vaccines contained cross-reactive epitopes between the parasite and the host (Kierszenbaum 1986).

Vervelde et al. (1993) recently labeled granules in the apical region of chick cecum epithelial cells with E.TEN 11M-2, a mab to a low-molecular *E. tenella* sporozoite surface protein, thus demonstrating that in vivo in the low molecular weight range of 18-19 kDa there are common cross-reacting epitopes between *E. tenella* sporozoites and host cells. In the present work we used mab B1C4 to localize further cross-reacting epitopes in *E. tenella* rhoptries, sporozoite cell nuclei, and nuclei of host primary chick kidney cells. Damian (1989) has referred to the presence of common antigenic epitopes between parasite and host cells as a phenomenon of "molecular mimicry". Such molecular mimicry leads to the induction of autoantibodies in man after infection with *Trypanosoma cruzi*. These considerations are also of major significance for the design of a subunit vaccine against coccidiosis, because inoculation with cross-reacting determinants could have unfavorable autoimmune consequences for the immunized chicken.

MATERIALS AND METHODS

Eimeria tenella (Houghton strain) was maintained in 3-week-old chickens (White Leghorn). Recovery, sporulation, and sterilization of oocysts were carried out as described previously by Raether et al. (1995), and protein fractions were prepared from purified sporozoites using the method of Dubremetz and Dissous (1980). For the immunization schedule we used a fraction enriched in micronemes, microsomal vesicles, ribosomes, and parts of rhoptries. BALB/c mice were given 7-8 immunizations intraperitoneally at 2-week intervals and by injections into the tail vein prior to fusion. Monoclonal antibodies (mabs) were obtained by fusion of x63-Ag 8.653 plasmacyioma myeloma cells with splenocytes of BALB/c mice according to Köhler and Milstein (1975). Rhoptry-specific antibody secreting hybrids were detected by indirect immunofluorescence antibody assay (IFAT) and the Western blot technique. Positive hybrids were subcloned by limiting dilution. Immunodetection of rhoptry proteins on nitrocellulose (Western blotting) was done after sporozoite-protein separation on a 12% SDS-polyacrylamide gel according to Laemmli (1970) and protein transfer to nitrocellulose according to Towbin et al. (1979). For immunodetection labeling with undiluted hybridoma supernatant was followed by labeling with anti-mouse IgG alkaline phosphatase (1:2,000, Sigma A5153) and bromochloroindolyl phosphate/nitro blue tetrazolium (BCIP/NBT) as substrate (Sigma). Molecular weight was evaluated by comparison with molecular weight markers (Pharmacia,

LMW kit). Immunoelectron microscopy was done as described previously (Entzeroth et al. 1993), using undiluted hybridoma supernatant, rabbit-anti-mouse IgG (1 : 1,000, Southern Biotech Association, RAM-6120-01), and protein-A-gold (1 : 100 in 2% PBSO). Production and cultivation of primary chick kidney cell (PCKC) cultures were performed according to Raether et al. (1995) on a Flexiperm slide system (Heraeus). Inununofluorescence assays (IFAT) of infected tissue cultures were done after 10 min of fixation in cold acetone using undiluted hybridoma supernatant for 1 hr at 37°C, washing three times in PBS, incubation in fluorescein-conjugated rabbit-anti-mouse IgG (Sigma F-0257), washing three times, and viewing under a Zeiss ultraviolet epifluorescence microscope (ICM 405) at 400 nm.

RESULTS

Immunoblot

Fusion of spleen cells from an immunized BALB/C mouse with tumor cells led to a rhoptry-specific monoclonal antibody (mab) A4C6 (Greif and Entzeroth, 1996) and to a cross-reacting antibody B1C4. The monoclonal antibody B1C4 recognized on the immunoblots of sporozoites a protein complex at 94, 66, 60, and 45 kDa as well as a protein band at about 20 kDa (Fig. 1j).

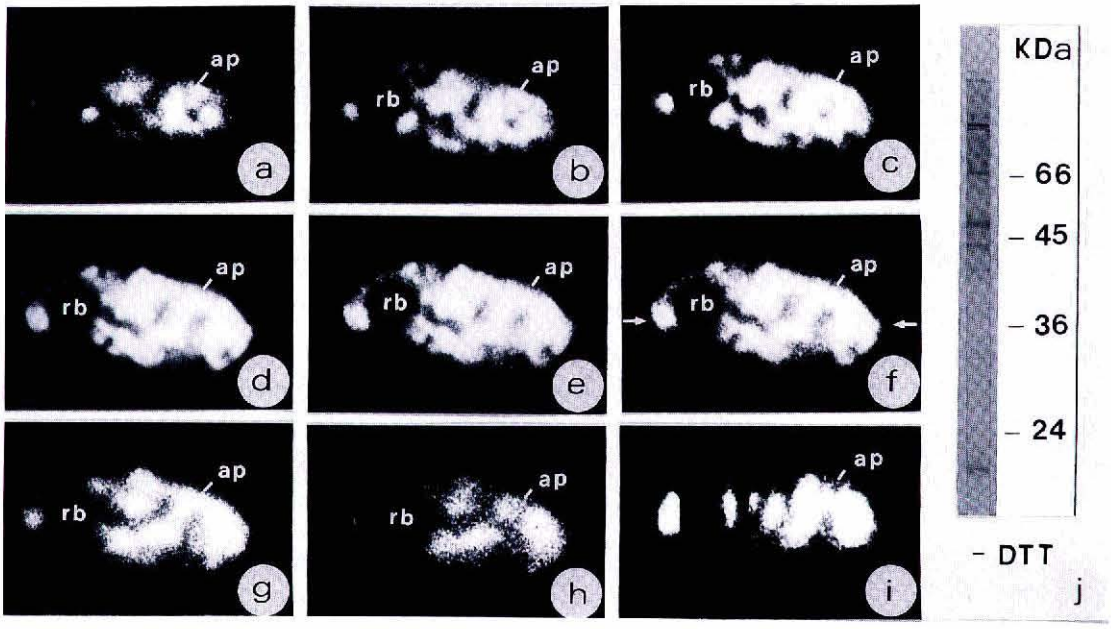

Fig. 1 Confocal sections through a sporozoite of *Eimeria tenella* after fixation and IFAT labeling with mab B1C4 (x 4300). a-h) Single sections 0.5 mm apart. i): xz-Section through sporozoite near the position of the white arrow in (f). j): Immunolabelling of sporozoite proteins by mab B1C4 after SDS-PAGE and Western blotting.

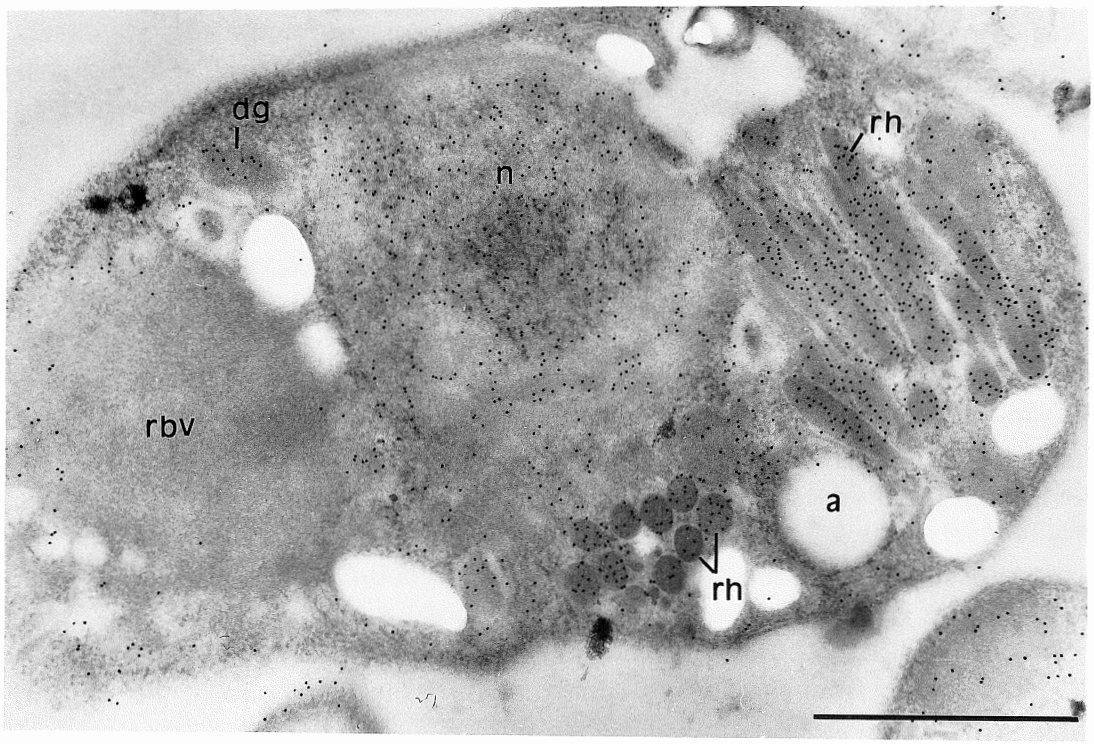

Fig. 2 Electron micrograph of an *Eimeria tenella* sporozoite. Immunolocalization of antigens with mab B1C4 and protein-A-gold in LR-White ultrathin section. Clear labeling of rhoptry (rh), nucleus (n), and dense granules (dg), no labeling of amylopectin (a) and the anterior refractile body (rbv). x 38,000 (bar=10 μm).

Immunofluorescence antibody assay (IFAT)

In confocal sections through fixed *E. tenella sporozoites,* mab B1C4 labeled granular structures in the inner cytoplasm (Fig. 1a-i). The sporozoite was labeled with granular fluorescence for up to 2/3 of its length. The labeled granules were concentrated over large areas of the apical and medial region of the sporozoite, but also behind the refractile body at the posterior pole. A confocal section through the sporozoite's xz-plane shows that the granules are not connected together in this plane (Fig. 1i).

Immunoelectron microscopy

The monoclonal antibody B1C4 labelled rhoptry ampoules tightly bundled together in the apical region of LR-White ultrathin sections of purified *E. tenella* sporozoites. These intersected rhoptries were circular in cross-section (Fig. 2). However, in addition to rhoptries, mab B1C4 also recognized the nuclear region

of the sporozoite and granular inclusions located between the nucleus and the refractile body. There was no labeling of the pellicle, the refractile body, and the amylopectin inclusions in the apical region of the sporozoite.

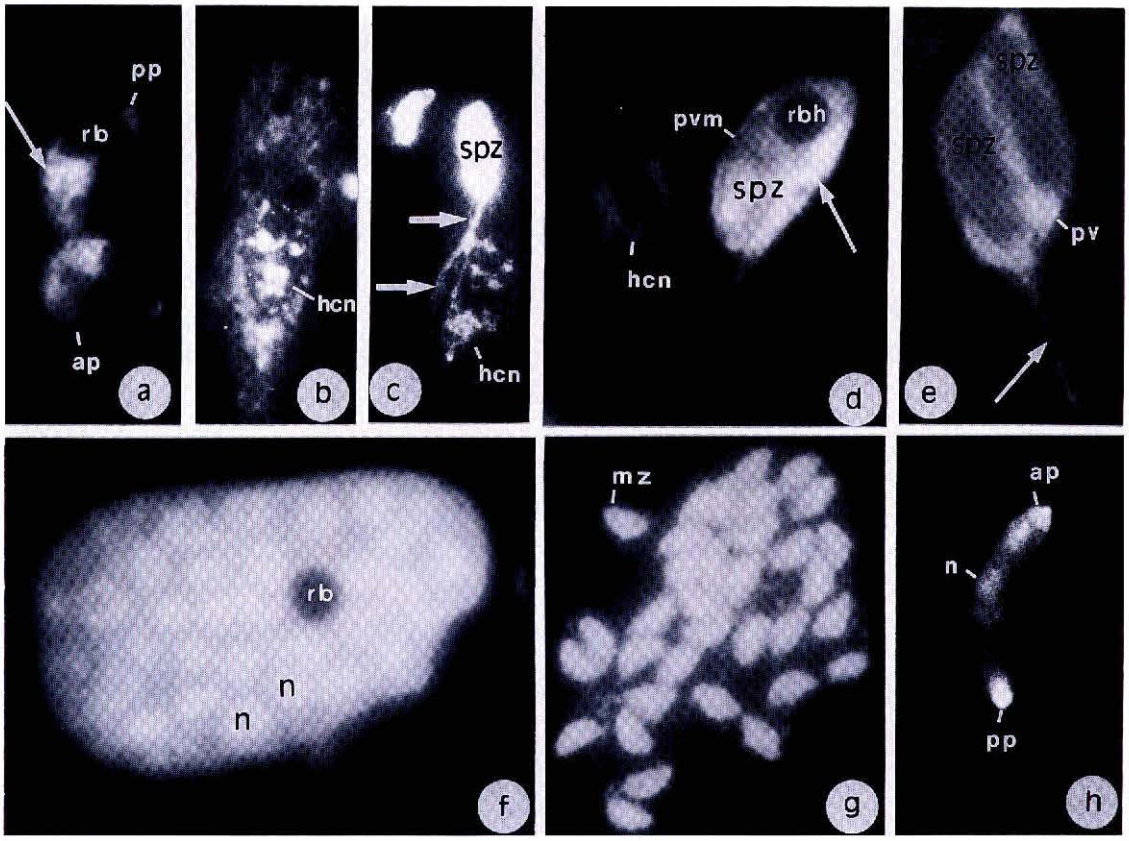

Fig. 3 IFAT labeling with B1C4 during parasite development in PCK host cells: a) *Eimeria tenella* sporozoite before invasion (x 3000). b) Noninfected PCK host cell (x 400). c) Intracellular sporozoite in PCK host cell 24 h p.i. (x 1400). d) Intracellular sporozoite 49 h p.i. (x 3000). e) Two intracellular sporozoites 24 h p.i. (x 3000). f) Multiplication of nuclei in the trophozoite stage 49 h p.i. (x 2200). g) Schizont I 72 h p.i. with merozoite stage I (x 2000). h) Free merozoite stage II 96 h p.i. (x 2000). ap = apical pole, hcn = host cell nucleus, mz = merozoite, n = nucleus, pp = posterior pole, pv = parasitophorous vacuole, pvm = parasitophorous vacuole membrane, rb = refractile body, rbh = posterior refractile body, spz = sporozoite.

Localization of cross-reacting antigens during invasion and development in primary chick kidney cells

Before cell invasion, B1C4 labeled granular structures in the apical and posterior pole as well as the nuclear region of purified *E. tenella* sporozoites (Fig. 3a). The antibody showed a clear cross-reaction with antigens in the nucleus of

uninfected PCK cells (Fig. 3b). 24 after infection there were intracellular sporozoites close to the nucleus of the host cell and B1C4 labeled a net-like connection between the sporozoite and the host cell nucleus (Fig. 3c). After cell invasion sporozoite proteins of apical region were secreted into the lumen of the parasitophorous vacuole (Fig. 3d, 3e) and were located between the intracellular sporozoite and the parasitophorous vacuole membrane in the form of protein aggregates. In addition to the parasitophorous vacuole membrane and the secreted protein aggregate, B1C4 labeled a filament extending from the latter membrane into the infected host cell cytoplasm (Fig. 3e). Morphogenesis of young schizonts with multiplication of nuclei in the trophozoite stage commenced 49 h after infection (Fig. 3f). Of the motile stages of the development cycle, stage I merozoites were labeled over their entire surface (Fig. 3g) and stage II merozoites at both apical poles and in the medial cytoplasmic region (Fig. 3h). Colour images of in-vitro development show the cross-reaction of mab B1C4 with the host cell nuclei of PCKC culture (Fig. 4a-c). The chromatin of the host cell nucleus in infected PCK cells was distinctly decondensed. The host cell nucleus thus appeared distended and became the size of a trophozoite. In neighbouring noninfected PCK cells the chromatin in the cell nuclei was clearly condensed.

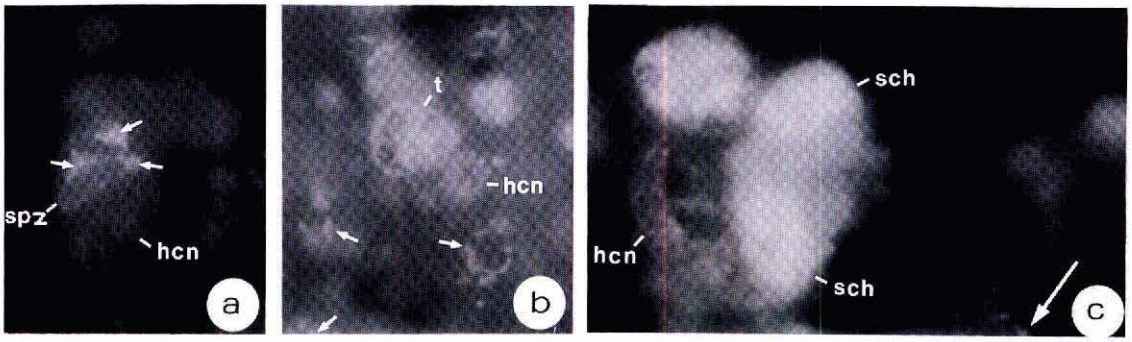

Fig. 4 IFAT labeling of an infected PCKC-culture with mab B1C4 shows the decondensation of infected host cell nucleus (hcn) (x 1000). The chromatin in the nucleus of noninfected cells is condensed (white arrow). a) Intracellular sporozoite spz = sporozoite, hcn = host cell nucleus, t = trophozoite, sch = schizont.

DISCUSSION

Antigens in rhoptries, granular inclusions, sporozoite cell nuclei and nuclei of primary chick kidney cells were localized with the aid of mab B1C4. This labeling shows that there are common antigenic epitopes between parasitic *Eimeria* sporozoites and the host cells.

The possible occurrence of molecular mimicry in coccidial infections has still hardly been investigated. Some metabolically or structurally important polypeptides sharing a high degree of homology with corresponding host cell polypeptides have been identified in *P. falciparum*, including heat-shock protein (Mattei et al. 1989), aldolase (Certa et al. 1988), tubulin (Sen and Godson 1990), and actin (Weseling et al. 1988), but the formation of autoreactive antibodies has only been demonstrated for single-strand DNA (Greenwood et al. 1970), heat-shock protein hsp70, and Ag 332, also localized in *P. falciparum* (Mattei and Scherf 1991). The involvement of autoreactive antibodies in autoimmune diseases of nerve tissue has been demonstrated unambiguously for *Trypanosoma cruzi* infections (van Voorkis and Eisen 1989). The pathological picture of *Plasmodium vinkei* malaria also seems to be caused by molecular mimicry of tumor necrosis factor (Clark et al. 1992). There is as yet no known evidence of the occurrence of autoimmune diseases after *Eimeria* infections.

In the immunoblot of sporozoites mab B1C4 recognized protein bands at 94, 66, 60, and 45 kDa. Recently Tomley (1994) compared electrophoretic profiles of rhoptries from several apicomplexan parasites and found they all had clusters of polypeptides that migrate in the 45- to 65- kDa range together with a number of high-molecular-weight proteins. However, B1C4 differs from mab A4C6 (Greif and Entzeroth I 996) by also labeling a protein band at about 20 kDa. Since A4C6 (specific labeling of rhoptries) and B1C4 (cross-reaction with rhoptries, nuclei, and host cell nuclei) only differ by the 20 kDa protein band, we conclude that the special property of clear nucleus labeling in sporozoites and in primary kidney cells is probably due to the 20 kDa protein.

Vervelde et al. (1993) recently localized a low-molecular polypeptide of 18-19 kDa in both sporozoites and host cells. The authors suspect that this protein is involved in the parasite-host cell interaction because in-vivo immunocytochemical methods only locate it in host cells which are specifically invaded by *E. tenella* sporozoites. By mimicry of host-specific polypeptides parasite stages can imitate ligands for which the host cells have specific receptors. Thus, the gene sequence of a sporozoite surface antigen in *Theileria annulata* is homologous with the extracellular matrix protein elastin (Hall et al. 1992). Elastin-like ligands can bind to the elastin receptors of macrophages, which are preferentially invaded by *Theileria parva* sporozoites. In malarial parasites there is a similarity between the extracellular matrix glycoprotein thrombospondin and the surface proteins of freely motile parasite stages (Goundis and Reid 1988, Robson et al. 1988). There are appropriate receptors for thrombospondin domains in the liver cells of the host. Molecular mimicry of cell adhesion molecules has also been discussed for *E. tenella* sporozoites (Tomley et al. 1991) and for *Sarcocystis* muris antigens

(Eschenbacher et al. 1993). At present it cannot be said whether the cross-reaction of rhoptry antigens with components of PCK host cell nuclei is responsible for an autoimmune reaction, evasion of the host' s immune response by the parasite (Damian 1989), or sporozoite invasion into the host cells (Vervelde et al. 1993). Since the nucleus region is a membrane-delimited intracellular compartment, cross-reaction with nuclear epitopes may serve to improve intracellular development of the parasite. Another possibility is that antigens recognized by mab B1C4 belong to the family of heat shock proteins (HSPs). This is a small group of highly conserved polypeptides that appear to be produced under conditions of stress, including protozoan infection (Nagasawa et al. 1992; Rondinelli 1994). HSPs appear primarily in the nucleus and have been found in Eimeria bovis sporozoites and merozoites (Robertson et al. 1988). Cloning and sequencing of B1C4 related antigens and sequence comparison will be necessary to prove this assumption.

Simultaneous labeling of host primary kidney cell nuclei and intracellular sporozoites reveals a remarkable change in the nucleus of infected host cells compared with noninfected cells. The nuclei of nonfected cells appear distended, as if the chromatin in the nucleus had become decondensed. This morphological impression may indicate activation of the cell nucleus by *E. tenella* sporozoites. Enlarged nuclei were found in PCK cells infected with schizonts in particular. Activation of the host cell nucleus could be connected with the high nutritional requirements of growing schizont stages. Parasites may exert a regulating influence on the host cell nucleus to prevent early division of the host cell, which would interfere with their reproduction and growth. Enlarged nuclei in infected cells have long been known morphologically for coccidia (Doran 1982).

Shortly after invasion sporozoites were located near the nuclei of infected cells, and started their further development in close contact with the nuclei. Adams and Bushell (1989) have also reported that *Eimeria veruaformis* sporozoites are frequently localized close to the host cell's perinuclear region. This region is particularly rich in cytoplasm and contains the microtubule-organizing centre of many eukaryotic cells. The localization of sporozoites observed by Adams and Bushell (1989) was connected with a change in the organization of the intracellular cytoskeleton caused by them.

In early stages, shortly after the invasion of PCK cells, intracellular sporozoites seem to be connected to the host cell nucleus by filamentous structures. Whereas at present there is no explanation for this kind of connection, it seems particularly interesting that Stewart et al. (1986) have demonstrated a visible connection between rhoptry proteins and the nuclear membrane of *P. falciparum* by electron microscopy: after fixation with tannic acid the authors

observed a membranous whorl on both rhoptries and the nuclear membrane of *P. falciparum*. The authors postulated that the nuclear membrane forms part of the rhoptry-microneme system in parasites. This, together with the endoplasmatic reticulum, would in turn form a continuous endomembrane system involved in cellular secretion. The authors discussed the development of rhoptries from the Golgi apparatus, which derives from the nuclear membrane.

In the present work mab B1C4 unambiguously demonstrated the presence of common epitopes in rhoptries and the nuclear region of sporozoites, as well as in the nuclear region of PCK host cells. Future investigations must clarify the functional connection between nuclear epitopes and rhoptry epitopes and the significance of these epitopes for intracellular development of apicomplexan parasites in vivo.

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