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Developmental changes in the circumsporozoite proteins of *Plasmodium berghei* and *P. gallinaceum* in their mosquito vectors

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Abstract The circumsporozoite (CS) protein covers the surface of the sporozoite of plasmodia. Its role in the development of the malaria parasite in mosquito vectors remains unknown. CS-epitope-containing proteins appear on undifferentiated oocysts on day 7 in *Plasmodium berghei* and on day 5 in *P. gallinaceum* as demonstrated by indirect fluorescence antibody tests using monoclonal antibodies directed against the CS-protein repeats. The three-dimensional distribution of the CS-epitope-containing proteins on oocysts was analyzed by confocal scanning laser microscopy. A strong antibody binding was found in patches around the oocysts of *P. berghei* and *P. gallinaceum*, and an accumulation of labeled proteins was found at the base of the oocysts of both species. In Western blots of infected midguts and salivary glands the antibodies recognized two peptides in the salivary glands but up to ten peptides in midgut extracts. The larger number of peptides recognized in midgut preparations might indicate breakdown products during the escape of the sporozoites from the oocyst and their migration on the midgut in the mosquito vector. The data indicate a possible involvement of the CS protein in an active migration process of the sporozoites in the mosquito vector.

Introduction

During the developmental cycle of the plasmodia, three invasive stages are found: the merozoites, which penetrate the erythrocytes; the ookinetes, which penetrate the mosquito midguts; and the sporozoites, which are unique due to their capacity to invade two types of target cells – the salivary glands in mosquitoes and the hepatocytes in the mammalian host (Garnham 1966; Sinden 1985). The sporozoites are covered by an immunodominant epitope, the circumsporozoite (CS) protein (Nussenzweig and Nussenzweig 1986), which has also been found in micronemes of mature blood stages (Cochrane et al. 1989).

Detailed studies on the formation and the function of the CS proteins of plasmodia in the mosquito vector have not been carried out. Immunoelectron microscopy studies have demonstrated the synthesis of CS protein in young oocysts at a time point before sporozoite differentiation (Hamilton et al. 1987; Nagasawa et al. 1987, 1988; Posthuma et al. 1987a, b; Golenda et al. 1990; Meis et al. 1992).By use of the indirect fluorescence antibody test (IFAT) and Western-blot analysis, the early synthesis of *Plasmodium falciparum* and *P. berghei* CS protein was confirmed in young undifferentiated oocysts (Boulanger et al. 1988; Simonetti et al. 1993). Most studies have been focused on the infectivity of midgut and salivary-gland sporozoites for the hosts. Some authors reported that midgut sporozoites were less infective than salivary-gland sporozoites because of immature CS proteins (Nussenzweig and Nussenzweig 1985). The time point of the sporozoite isolation was crucial for the ability of sporozoites to infect the vertebrate host (Ball and Chao 1961; Schneider 1968; Walliker and Robertson 1970; Daher and Krettli 1980, 1987). Touray et al. (1992) demonstrated that sporozoites from oocysts were capable of infecting salivary glands of uninfected female mosquitos, whereas sporozoites isolated from salivary glands did not reinvade the salivary glands of uninfected females. The authors found no qualitative difference using IFAT between sporozoites derived from oocysts and salivary glands.

In the present study, we analyzed the CS proteins of *P. berghei* (rodent malaria) and *P. gallinaceum* (avian malaria) using IFAT, Western-blot analysis, and confocal scanning laser microscopy (CSLM) to detect potential

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differences between midgut and salivary-gland sporozoites that might help to explain possible functions of the CS protein in the mosquito vector.

Materials and methods

Parasites

Plasmodium berghei (ANKA strain) was cyclically transmitted in *Anopheles stephensi* maintained at 21° C and 80% humidity (Vanderberg and Gwadz 1980). The infection of the mosquitoes was examined at 10 days after the infective blood meal by counting the number of oocysts per midgut and around day 20 by checking the presence of sporozoites in the salivary glands. *P. gallinaceum* (Liverpool strain) was maintained in chickens and infected *Aedes aegypti* maintained at 28° C and 80% relative humidity. The infection of the mosquitoes was checked at day 5 by the presence of oocysts and at day 10 for the presence of sporozoites in the salivary glands. Infected midguts and salivary glands were dissected, isolated in phosphate-buffered saline (PBS), and stored at −20° C until use.

Monoclonal antibodies

P. berghei

A supernatant of the monoclonal antibody (mAb) NBS1 (IgG3) was used, produced by immunization of mice with radiation-attenuated salivary-gland sporozoites and directed against the CS protein of *P. berghei* as verified by the induction of a strong circumsporozoite precipitation (Y Charoenvit, unpublished data).

P. gallinaceum

Ascitic fluid of mAb N8B4H2 (842), raised against oocyst sporozoites, containing IgG1 antibodies recognizing the CS protein of *P. gallinaceum* was used (Krettli et al. 1988; Rocha et al. 1990).

Indirect fluorescence antibody test

P. berghei- and *P. gallinaceum*-infected midguts were analyzed on days 7–19, i.e., at 5–12 days after the infective blood meal. Six infected mosquito midguts were placed on each well of multispot antigen slides (Flow Laboratories, Irvine, Scotland) and kept at −20° C until use. The slides were fixed with methanol for 10 min at 4° C and, after careful washing of the wells with drops of PBS containing 0.5% bovine serum albumin (PBS/BSA), the slides were incubated for 20 min at 37° C with the mAbs (diluted 1/50 for *P. berghei* and 1/100 for *P. gallinaceum*). The slides were washed with PBS/BSA and incubated for 20 min at 37° C with 50 µl of a 1/50 dilution of fluorescein isothiocyanate (FITC)-labeled goat anti-mouse/IgG (Kirkegaard and Perry Laboratories, Gaithersburg, Md.). After the final wash, the slides were dried, mounted in 50% glycerol in PBS, and examined under a UV microscope (Wild Leitz Ltd, Heerbrugg, Switzerland).

Confocal scanning laser microscopy

The midgut samples were processed as described for the IFAT analysis. *P. gallinaceum* preparations were studied at day 9 and *P. berghei* samples, at day 17 after the infective blood meal. To improve the resolution, the slides were mounted in 50% glycerol in PBS with 2.5% DABCO [1,4-diazabicyclo (2.2.2.) octane, Fluka]. An Axiovert 35M inverted microscope (Zeiss, Oberkochen, Germany) coupled to the CSL (Bio Rad Laboratories, MRC 600, Richmond, Calif.) was used to observed the samples with a 40× objective. Each optical section was of 0.5-µm thickness.

Western-blot analysis

Starting on the day of infection and until day 19 postinfection, the midguts and salivary glands of 20 mosquitoes were dissected for each time point and stored at −20° C. The midguts and salivary glands were dissolved in sample buffer, separated via 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto nitrocellulose membranes, rinsed with a washing buffer [50 m*M* TRIS, 140 m*M* NaCl, 5 m*M* ethylene diamine tetraacetic acid (EDTA), 0.05% NP40, 0.25% gelatin (pH 7.4)], and incubated at room temperature for at least 1 h in a blocking buffer (washing buffer containing 1% nonfat dry milk; Rapilait, Migros, Switzerland). Incubation with mAbs diluted in blocking buffer (1/1000 for *P. gallinaceum* and 1/30 for *P. berghei*) was carried out at room temperature overnight. The nitrocellulose membranes were washed, incubated for 2 h at room temperature with goat anti-mouse/IgG conjugated with peroxidase (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) and diluted 1/1000 in blocking buffer, and finally incubated in the peroxidase substrate (3 mg of 4-chloro-1-naphthol/ml of methanol and 0.02% H_2O_2 in PBS; Towbin and Gordon 1984).

Results

Immunofluorescence and confocal microscopy

In *Plasmodium berghei* the CS protein was detectable on oocysts of the mosquito midgut as early as at 7 days after the infective blood meal (Fig. 1a). The antibody binding was diffusely distributed around the oocyst (Fig. 2b). In mechanically ruptured oocysts, sporozoites could be labeled from day 7 onward. Inside intact oocysts the sporozoites were not labeled and could be detected only at the beginning of their release at day 10 (data not shown). Sporozoites were detectable on the midgut up to day 19. In confocal microscopy carried out on midgut preparations at 17 days postinfection, the surface of oocysts was covered by spots of labeled proteins containing CS epitopes. Some antibody binding was also detected inside the oocyst and, especially, on the basal lamina of the midgut epithelium (Fig. 3).

In *P. gallinaceum* the binding of antibodies to the oocysts could be detected as early as at day 5 postinfection, and the sporozoites were released at day 7 (Fig. 1b). The antibody binding was strongest around the oocyst (Fig. 2a). Confocal microscopy showed that the antibody binding was localized mostly at the base of oocysts at 9 days after the infective blood meal. Sporozoites were found outside the oocyst on the midgut, but none could be detected inside oocysts (Fig. 4).

At an early differentiation stage of both malaria species, oocysts that bound antibodies occurred concurrently with those that did not bind antibodies (Fig. 2a; data not shown). *P. gallinaceum* oocysts developed faster than those of *P. berghei*. Sporozoites of *P. berghei* could be detected on midguts of *Anopheles stephensi* much longer than could those of *P. gallinaceum* on midguts of *Aedes aegypti*.

Western-blot analysis

The numbers and molecular weights of the different proteins recognized by antibodies directed against the repet-

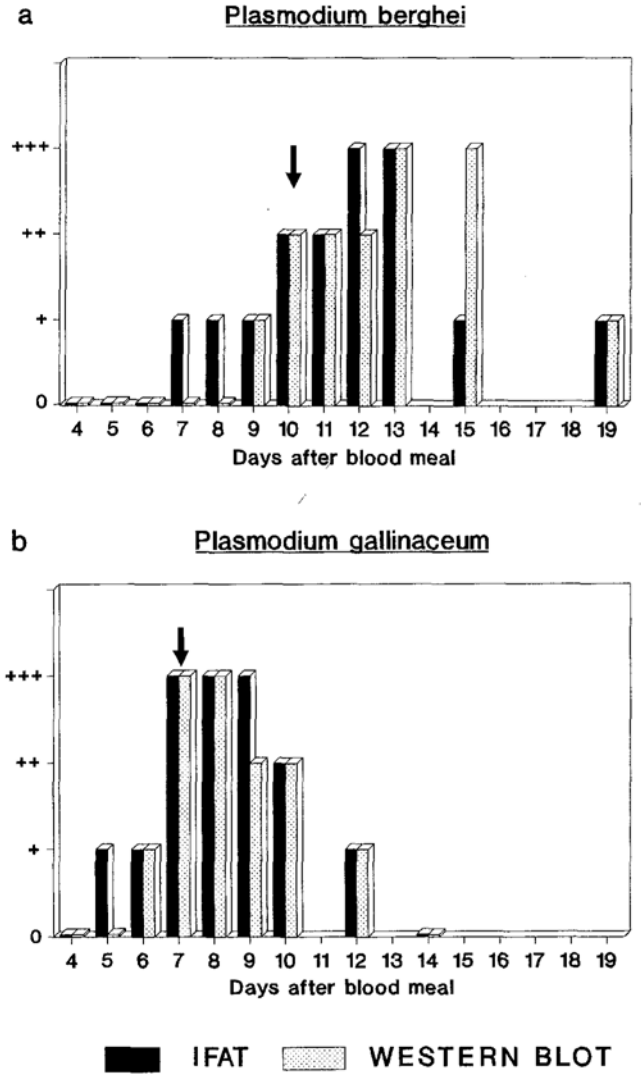

Fig. 1a,b Kinetics of the appearance of the CS protein on midguts of **a** *Plasmodium berghei* and **b** *P. gallinaceum*-infected mosquitoes as determined using IFAT and Western-blot analysis. *Arrows* indicate the onset of sporozoite release. The relative intensity of antibody binding is recorded as low (+), medium (++), and high (+++)

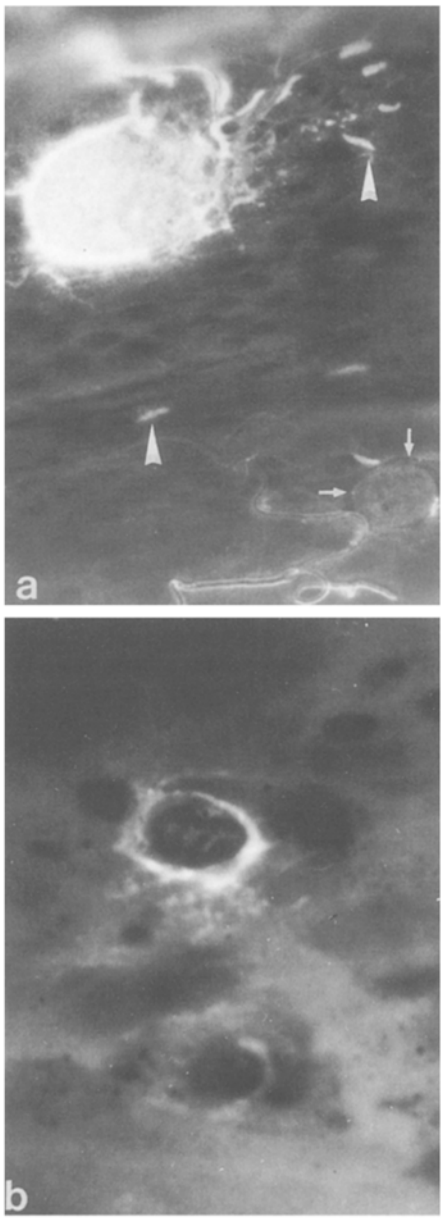

Fig. 2a,b MAb binding on midgut oocysts of **a** *P. gallinaceum* and **b** *P. berghei* from mosquitoes at 9 days after the infective blood meal. NBS1 binding is visible at the periphery of the *P. berghei* oocyst (magnification, ×1200), and 842 antibodies have reacted over the entire oocyst of *P. gallinaceum* (magnification, ×890) as well as with sporozoites on the midgut (*arrowheads*). A young oocyst without antibody binding is indicated by *small arrows*

itive CS epitopes were determined at different time points after the infective blood meal. CS epitopes were detectable 1–2 days later than with IFAT (Fig. 1).

Proteins with CS epitopes of *P. berghei* could be detected in midgut preparations at 9 days after infection, with labeling increasing in intensity until day 15, and could be observed as late as on day 19 after the infection. At least 7 peptides with relative molecular weights (Mr) ranging from 42 to 59 kDa were present at day 13. The most prominent peptides were at 48 and 59 kDa (Fig. 5a). In salivary-gland preparations, only two CS-epitope-containing proteins of 48 and 59 kDa were detectable starting on day 10, with the labeling increasing in intensity up to day 19 (Fig. 5c), the last time point assayed.

In *P. gallinaceum*-infected *Aedes aegypti*, proteins reacting with mAb 842 could be detected in midgut preparations as early as at 6 days after the infective blood meal (Fig. 6a). Three strong proteins of 67, 73, and 78 kDa, respectively, and a weaker one at 43 kDa were detectable at 8 days postinfection; the intensity of the bands quickly decreased such that in extracts examined at 12 days after infection, practically no peptide could be detected. In salivary-gland preparations, only two polypeptides of 67 and 43 kDa could be detected from days 7 to 17 (Fig. 6c).

The numbers of CS-epitope-containing proteins detected in midguts infected with *P. berghei* or *P. gall-*

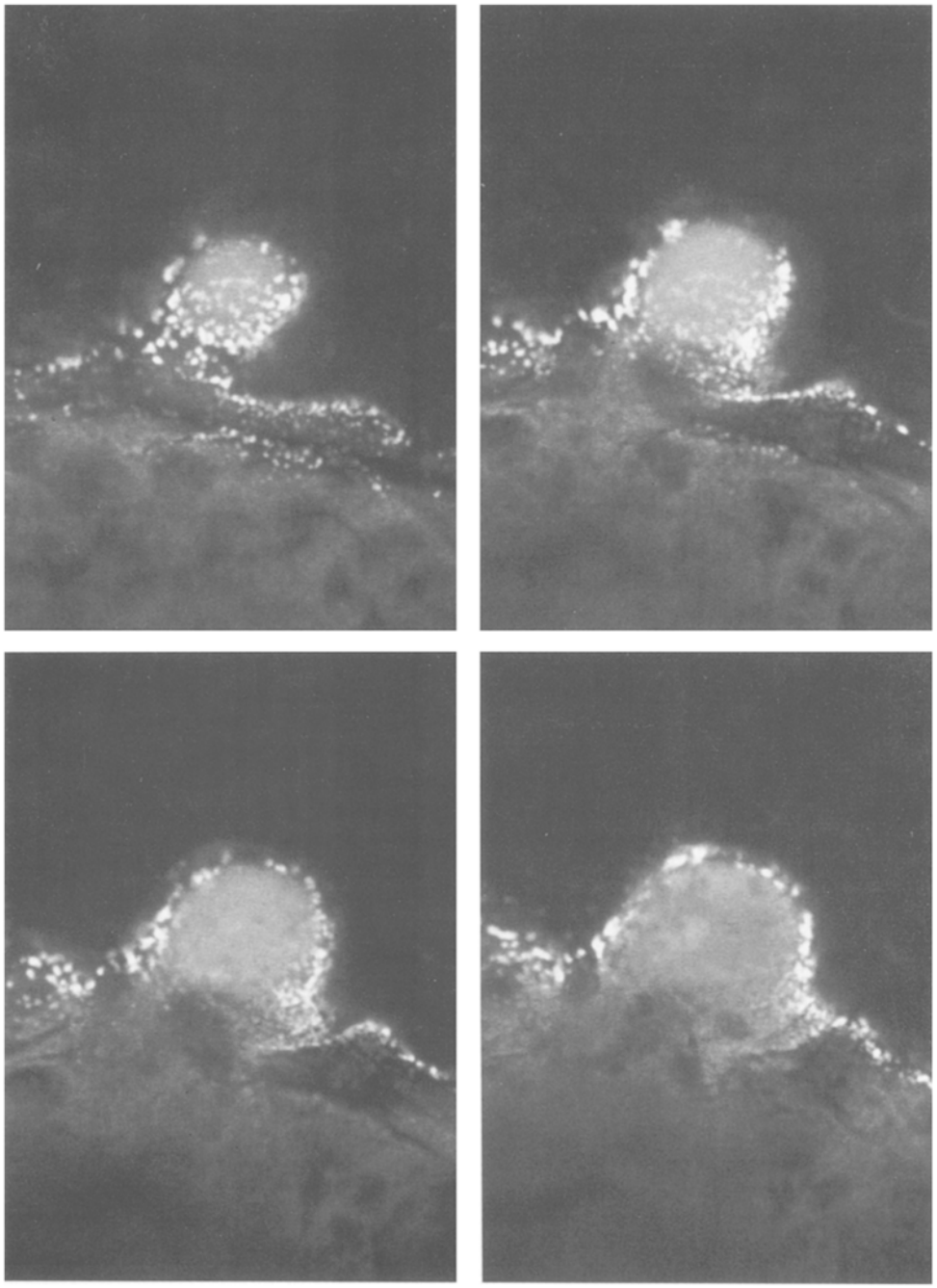

Fig. 3 CSLM of a day-17 *P. berghei* oocyst incubated with mAb NBS1 and FITC-labeled goat anti-mouse IgG. CS epitopes are localized in discrete patches around the oocyst and on the midgut. In all, 4 pictures were selected from 13 optical sections during movement of the specimen from the top of the oocyst in 0.5-µm steps through the focal plane of the 40× optical objective lens

inaceum were greater than those observed in infected salivary glands. To test whether these differences were caused by proteolytic degradation of the protein during preparation, fresh extracts were prepared with 1 m*M* of the protease inhibitor phenylmethylsulfoxide (PMSF). Surprisingly, in *P. berghei*-infected midguts a clear peptide ladder of at least ten peptides could be detected (Fig. 5b), and in *P. gallinaceum*-infected midgut preparations the 43-kDa polypeptide was better preserved with than without PMSF (Fig. 6b). Such differences were not detectable in infected salivary glands (Fig. 5, 6d).

Discussion

The presence of CS-epitope-containing proteins on the oocysts before sporozoite differentiation is in agreement with immunoelectron microscopy studies performed on different plasmodia (Hamilton et al. 1987; Nagasawa et al. 1987, 1988; Posthuma et al. 1987a, b). Early experiments on the development of oocysts (Sinden and Strong 1978; Boulard et al. 1983) suggested an intensive protein synthesis beneath the cyst wall in the young oocysts leading to a fibrous layer at the surface of the oocyst. This layer might correspond to the intensely stained material around the oocysts described in this work. The plasmalemma and the

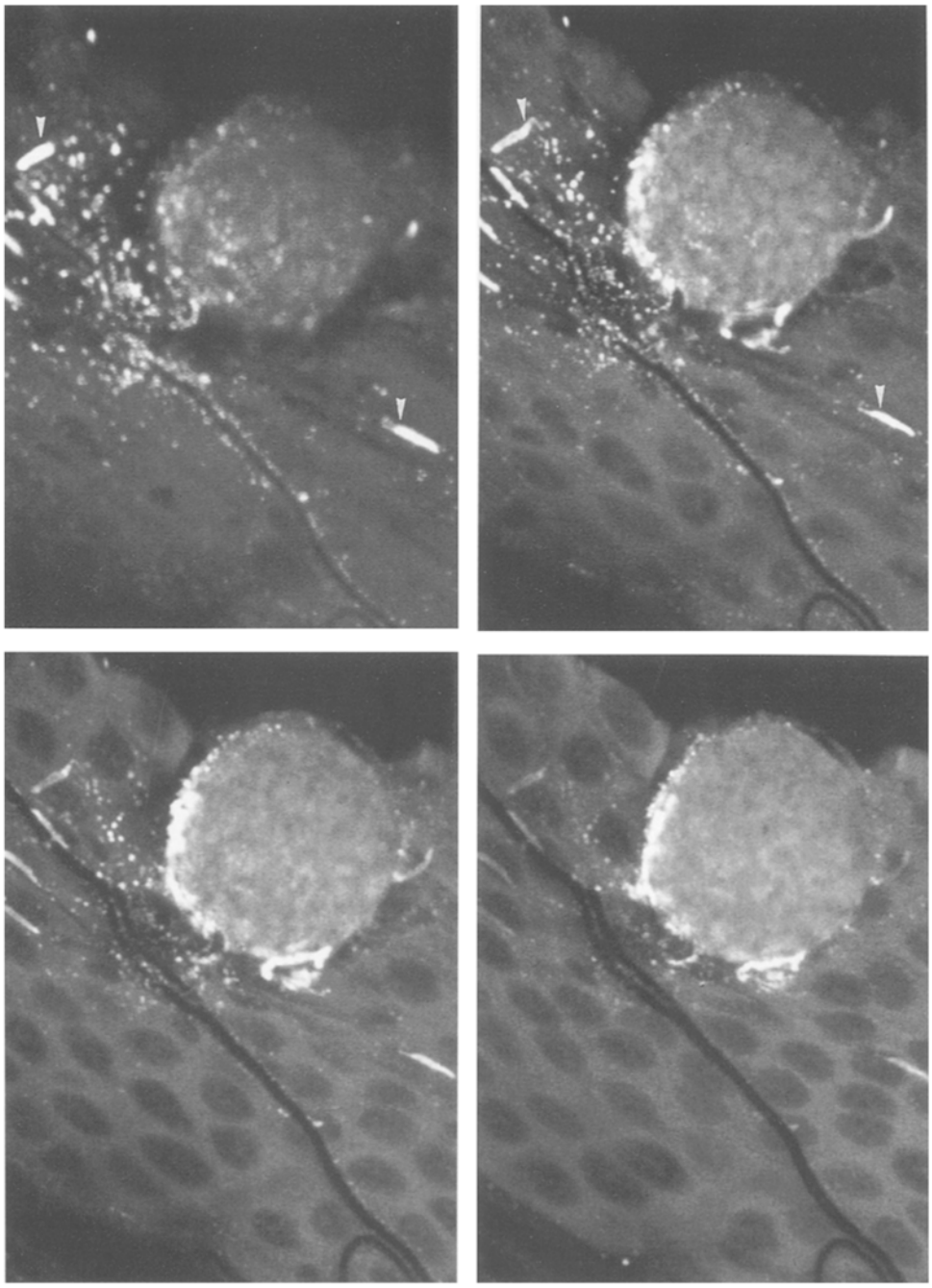

Fig. 4 Immunoreactivity of a day-9 *P. gallinaceum* oocyst as determined using CSLM. The binding of mAb 536 is concentrated at one side of the oocyst, where sporozoites (*arrowheads*) are visible on the midgut. In all, 4 pictures from a total of 16 optical sections were selected during movement of the specimen from the midgut surface to the top of the oocysts in 0.5-µm steps through the focal plane of the 40× objective lens

rough endoplasmic reticulum of the oocysts were shown to be heavily labeled (Hamilton et al. 1987; Nagasawa et al. 1987, 1988) and, at a later stage, sporozoites inside the oocyste were also found to be labeled.

We could detect antibody binding to sporozoites inside the oocysts only when the oocyst membranes had been mechanically ruptured allowing the antibodies to penetrate the oocyst sufficiently. Using *Plasmodium berghei*, Simonetti et al. (1993) also showed a preferential labeling of the oocyst periphery at 10 days postinfection. In *P. gallinaceum* the antibody binding was localized at the surface of the oocysts and the labeled proteins were concentrated mainly at a site close to the midgut, where sporozoites were released from the oocyst (Sinden and Strong 1978). The antibody-binding pattern suggests that CS protein is deposited by sporozoites leaving the oocysts (Sinden 1974; Rastogi et al. 1988). For confocal microscopy, midgut preparations were used at time points when most of the sporozoites had left the oocyst; hence the CS-protein spots found, especially on the oocysts of *P. berghei*, could represent the places where sporozoite passage occurred.

Since the synthesis of CS-epitope-containing protein takes place very early in the maturation of the parasite, it can be expected that the proteins play an important role in the development of the sporozoites in the mosquito

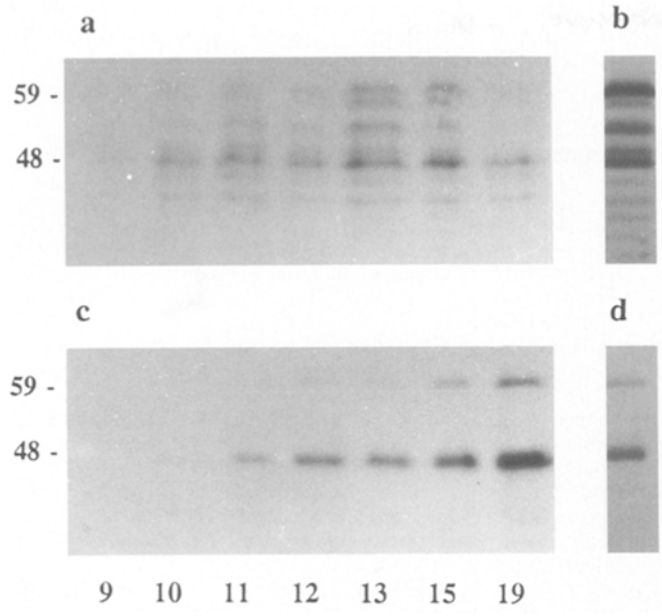

Fig. 5a–d Western-blot analysis of *P. berghei*-infected **a** midgut and **c** salivary-gland extracts from day 9 until day 19 after the infective blood meal. MAb NBS1 was used at a dilution of 1/30. **b**, **d** Infected midguts (from day 13) and salivary glands (from day 16), isolated in PBS in the presence of 1 m*M* of PMSF. Numbers at the *left side* of the figure indicate the molecular weight of the CS protein in kilodaltons.

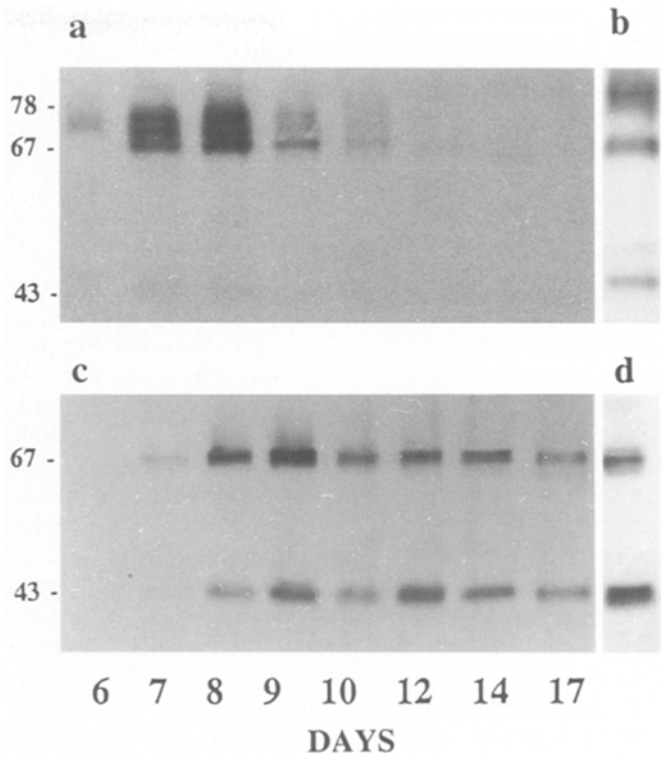

Fig. 6a–d Western-blot analysis of *P. gallinaceum*-infected **a** midgut and **c** salivary-gland extracts from day 6 until day 17 after the infective blood meal. MAb 842 was used at a dilution of 1/1000. **b**, **d** Infected midguts (8 days postinfection) and salivary glands (16 days) isolated in PBS in the presence of 1 m*M* of PMSF. Numbers at the *left side* of the figure indicate the molecular weight of the CS protein in kilodaltons

vector. The involvement of these proteins in the motility of sporozoites as well as the need for a substrate for gliding motility has been suggested (Stewart and Vanderberg 1988, 1991; King 1988). After leaving the oocysts the sporozoites could bind to specific receptors present on the midgut wall, which would allow their active migration and a concomitant release of the CS proteins. Similar patches of CS protein of *P. falciparum* have been observed on infected midguts (Boulanger et al. 1988), and this finding as well as the observation of trails of immunocomplexes behind sporozoites when the CSP reaction is performed in suspension (Boulanger et al. 1988; Stewart and Vanderberg 1988) could be parts of a gliding mechanism. Posthuma et al. (1987a,b) observed the binding of antibody directed against the CS protein on the basal lamina of the salivary glands during penetration of the sporozoites. Golenda et al. (1990) described the presence of small immunoreactive vesicles on the surface of salivary glands after sporozoite invasion, which could be deposits of CS-protein breakdown products similar to the small particles detected on the surface of oocysts and midgut epithelia.

That CS-epitope-containing proteins were detected with Western blotting 2 days later than with IFAT is probably due to the different sensitivities of the techniques. The molecular weights of the proteins of each *Plasmodium* species differed somewhat from those previously published (Table 1), but such variations have previously been observed and are a property of the SDS-PAGE technique used (Vermeulen 1983).

P. berghei- and *P. gallinaceum*-infected midguts and salivary glands showed striking differences in their pro-

Table 1 Comparison of the molecular weights of the circumsporozoite proteins of different *Plasmodium* species

Plasmodium	Molecular weights of the CS protein		
	In this work	In the literature	
P. gallinaceum		[a]	
From midgut	43 kDa 67 kDa 73 kDa 78 kDa	64 kDa 76 kDa	
From salivary glands	43 kDa 67 kDa	64 kDa 76 kDa	
P. berghei		[b]	[c]
From midguts	30–48 kDa 50 kDa 54 kDa 56 kDa 59 kDa	44 kDa 52 kDa 54 kDa	28–48 kDa 50 kDa 60 kDa
From salivary glands	48 kDa 59 kDa	44 kDa 52 kDa 54 kDa	28–48 kDa 50 kDa 60 kDa

[a] Krettli et al. 1988; Rocha et al. 1990
[b] Yoshida et al. 1980, 1981; Aikawa et al. 1981; Santoro et al. 1983; Vermeulen 1983; Cochrane et al. 1984
[c] Simonetti et al. 1993

tein patterns recognized by CS-specific antibodies, which were never detected in *P. falciparum* preparations under identical experimental conditions (Boulanger et al. 1988). It is possible that the CS-epitope-containing proteins of *P. falciparum* are differently processed in the mosquito vector. The differences found in *P. berghei* and *P. gallinaceum* might reflect potential developmental changes during sporozoite maturation. Isolation of *P. berghei*-infected midguts in PMSF did not result in a reduction in the numbers of proteins recognized by mAb NBS1 but rather enhanced the presence of a ladder of peptides. A similar ladder of CS-epitope-containing proteins was recently described by Simonetti et al. (1993). It is not clear why this ladder was also present in their salivary-gland preparations, as we have never been capable of detecting it in pure salivary-gland preparations. The presence of these peptides on midgut preparations suggests again that they might be formed during the migration of the sporozoites on the midgut epithelium.

Differences between midgut and salivary-gland sporozoites have been amply described in the literature. At the ultrastructural level, Sinden and Garnham (1973), Sterling et al. (1973) and Turner (1981) observed modifications of rhoptries and micronemes, both involved in a secretory-excretory function, suggesting that some material could be expulsed from the rhoptries during the process of sporozoite migration and penetration into the salivary glands. Dubremetz et al. (1979) compared the membranes of the two sporozoite populations using the freeze-fracture technique. They also noticed variations in the ultrastructure of the sporozoite membranes between the two populations. It was recently shown that only *P. gallinaceum* sporozoites isolated from oocysts could invade the salivary glands of uninfected mosquitoes (Touray et al. 1992). Nonetheless, Rocha et al. (1990) characterized the *P. gallinaceum* CS proteins extracted from sporozoites of oocysts and salivary glands; they reported equal numbers and sizes for the CS proteins of both extracts. However, on the basis of our data it is possible to speculate that the larger family of CS-epitope-containing proteins found on infected midguts are in part molecules that are released and processed during migration of the sporozoites.

In contradiction to the widely accepted theory that the sporozoites are released into the hemolymph via ruptured oocysts, we suggest that a significant proportion of sporozoites undergo a migratory phase on mosquito tissues, as seen especially on the midgut preparations examined in the present work. Whether this population is responsible for the salivary-gland penetration remains to be shown, but if the sporozoites were to reach the salivary glands, they would stop active migration and the production of processed CS protein would no longer occur. This would explain why generally only a subset of two CS proteins is found in sporozoites of the salivary glands of *P. berghei* and *P. gallinaceum*.

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