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Formation of the circumsporozoite protein of *Plasmodium falciparum* in *Anopheles stephensi*

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Summary

The place and time of synthesis of the circumsporozoite protein of *P. falciparum* was analysed with a monoclonal antibody directed against the $(NANP)_3$ repetitive epitope of the CS protein. By using an indirect fluorescent antibody test the epitope could be detected on the oocyst 7 days after infection and 3 days before the appearance of mature sporozoites. Using the Western blot technique, 3 polypeptides from midgut preparations were recognized by the $(NANP)_3$-specific monoclonal antibody from the day 9 of infection onwards. The circumsporozoite precipitation reaction could be induced in sporozoites from either the midgut or the salivary glands 11 days after a blood meal. A similar reactivity of midgut-sporozoites and salivary gland-sporozoites was observed with antisporozoite antisera. Not all sporozoites recovered from the midgut showed a precipitation reaction.

Key words: *Plasmodium falciparum;* sporozoites; circumsporozoite protein; Western blot analysis; indirect fluorescent antibody test; circumsporozoite precipitation reaction.

Introduction

The sporozoite of *Plasmodium falciparum,* the infectious stage of human malaria, develops in mosquitoes of the genus *Anopheles* sp. The sporogonic cycle begins after an infective blood meal by the fusion of a microgamete with a macrogamete. The resulting zygote becomes a motile ookinete which passes through the peritrophic membrane and the epithelial cell layer of the midgut (Freyvogel, 1966). The oocysts develop on the outside of the midgut and release

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sporozoites into the hemolymph, from which they migrate to the thorax and invade the salivary glands (for review see Sinden, 1984, 1985).

The infectivity of the sporozoites from the midgut and the salivary glands has been studied previously (Aikawa et al., 1981; Nussenzweig and Nussenzweig, 1985, 1986). It was suggested that the sporozoites from the salivary glands are more likely to be infectious because they are covered uniformly with the circumsporozoite protein (CS protein), whose immunodominant repetitive epitope is the $(NANP)_3$ (Zavala et al., 1985).

There are contradictory reports about whether the midgut sporozoites can also be infectious and to what extent the surfaces of sporozoites from the oocysts and from the salivary glands are identical. Infection of different vertebrate hosts with midgut sporozoites directly isolated from mosquitoes (Shute, 1943; Yoeli and Most, 1960; Vanderberg, 1975; Daher and Krettli, 1980) or after in vitro maintenance (Ball and Chao, 1961; Schneider, 1968; Walliker and Robertson, 1970) has been obtained at a time when no sporozoites could be found in the salivary glands. The infectivity has been shown to increase during the migration of sporozoites to the salivary glands (Vanderberg, 1975), but it is not known whether this is caused by an increased number of sporozoites. Recent immunoelectron-microscopical studies performed on midgut-preparations of different *Plasmodium* species (Hamilton et al., 1987a, b; Nagasawa et al., 1987; Posthuma et al., 1987) demonstrated the synthesis of the CS protein within the developing oocysts.

We have extended these initial studies by a detailed analysis of the development of the CS protein by using a monoclonal antibody to detect the appearance of the CS protein on the basis of the IFAT and Western blot techniques and the CS precipitation assay.

Material and Methods

Plasmodium falciparum (strain NF 54) was cultivated in an automatic incubator (Ponnudurai et al., 1982). Mosquitoes of the genus *Anopheles stephensi* were grown in an insectary at 26° C with 80% relative humidity. Three to 4 days after emergence, the female mosquitoes were fed on infected blood through a synthetic membrane (parafilm) (Ponnudurai et al., 1982). Seven days after the infective blood meal, the mosquito midguts were dissected out and the infection rate was determined by recording the presence of oocysts. Fifteen days after the blood meal, the salivary glands were dissected out and checked for the presence of sporozoites. The extent of the infection was always greater than 90% with an average of 30 oocysts per mosquito. From each mosquito, the midgut and the salivary glands were dissected in PBS (phosphate buffered saline) and stored at – 20° C for further use in the IFAT and Western blot experiments.

Two Balb/C mice were immunized by 2 intraperitoneal injections, 4 weeks apart, with frozen sporozoites from salivary glands (300,000 sporozoites in Freund's complete adjuvant and 500,000 sporozoites in Freund's incomplete adjuvant). Two days before the fusion, 2 intravenous boosters were given each with 100,000 sonicated sporozoites. Hybridomas were produced according to the technique of Koehler and Milstein (1975). The Ig classes of the 5 monoclonal antibodies obtained were determined by the Ouchterlony precipitation assay using clone-specific rabbit antisera (Nordic Immunologicals). The ELISA was carried out using established procedures (Etlinger et al., 1987). In the present study the monoclonal antibody sp_3/B_4 was used.

At different times after infection, the CSP reaction was carried out by using 10 microliters of freshly isolated sporozoites (either from the midgut or from the salivary glands) in PBS mixed with 10 microliters of ascitic fluids containing the antibody. The precipitation reaction was directly observed by phase contrast-microscopy after 5 min at room temperature, and graded +++, ++, +, according to the intensity of the reaction (Vanderberg et al., 1969).

For the Western blot, samples were used with either 5 infected midguts or 5 sets of salivary glands, dissected from the same mosquitoes. The samples were boiled for 5 min in reducing sample buffer (0.01 M Tris-HCl pH 6.8; 5% 2-mercaptoethanol; 10% glycerol; 2.3% SDS and 0.01% Bromophenol blue). Whole mosquitoes, infected or uninfected, were ground in reducing sample buffer, boiled and centrifuged. The supernatants of these total extracts were used as positive and negative controls. The electrophoresis was carried out on a 10% SDS-PAG and the samples then transferred onto nitrocellulose (Burnette, 1981). The nitrocellulose foil was washed between every incubation step (Tris 50 mM; NaCl 140 mM; EDTA 5 mM; NP-40 0.05%; gelatine 0.25% pH 7.4). Unspecific binding sites were blocked by incubation for 2 h in the same buffer supplemented with 1% BSA. The incubation with the monoclonal antibody was carried out overnight at room temperature in a 1/1000 dilution in blocking buffer. The binding of the antibody was finally revealed by incubation for 2 h with goat antimouse antiserum (IgA+IgG+IgM/peroxidase, Kirkegaard+Perry Laboratories, Gaithersburg, Maryland) or rabbit antimouse IgG(h+l)/peroxidase (Nordic Immunologicals Lab. Tilburg, The Netherlands), both used at a dilution of 1/1000 in blocking buffer. The colour was developed by the addition of 0.2 mg/ml of 4-chloro-1-naphthol and 0.005% H_2O_2 in PBS.

The titration of the monoclonal antibodies was carried out using the IFAT procedure with sporozoites from salivary glands, counted in a Neubauer chamber and diluted in PBS to give 2000 sporozoites per 5 μl. Drops of 5 μl were applied to the 12 wells of multitest slides, air dried at room temperature and stored at –70° C. The sporozoites were fixed with acetone before use. The IFAT was carried out as described by Young et al. (1985). The sera were diluted in PBS with 1% BSA and 10 μl of the corresponding dilutions were applied to the wells containing sporozoites. After 30 min incubation in a moist chamber at 37° C, the slides were washed 3 times with PBS-BSA. Fluorescein-conjugated rabbit anti-mouse IgG antiserum was then added at a dilution of 1/50 and the slides were incubated for another 30 min. After an additional wash in PBS, the samples were mounted in 50% glycerol in PBS.

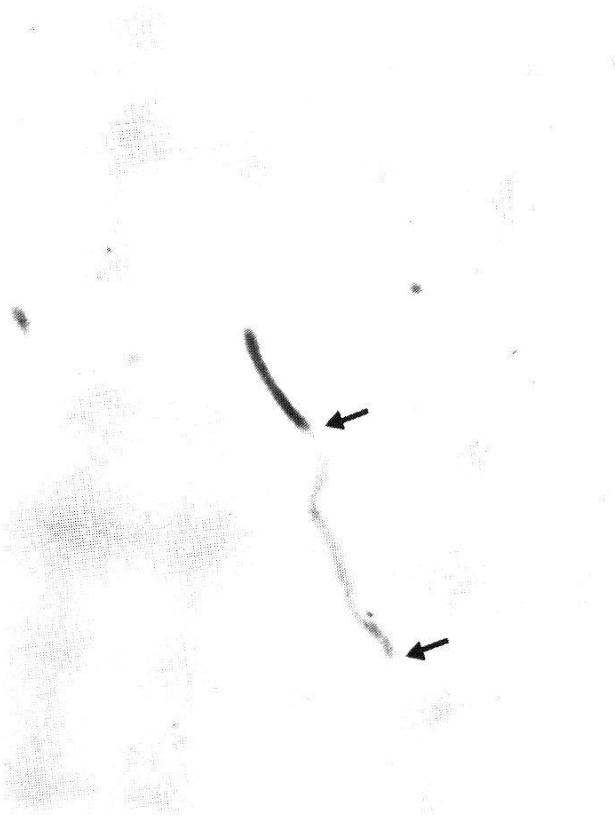
For the suspension-IFAT, 50 μl of salivary gland sporozoites were mixed with 50 μl of monoclonal antibodies and incubated at room temperature for 45 min. The suspension was washed twice with PBS by centrifugation. Spots of 10 μl were applied to the wells of multitest slides (Flow laboratories, Irvine, Scotland) and air-dried. Some of them were fixed with acetone. The slides were then incubated with rabbit antimouse FITC-conjugated antiserum (Nordic Immunologicals Lab., Tilburg, The Netherlands) at a dilution of 1/50 for 20 min at 37° C. After an additional PBS-wash, the samples were mounted in 50% glycerol in PBS. For the detection of the appearance of the CS protein in the midgut, multiple slides were prepared with 6 midguts for each day assayed. The rest of the protocol was the same as above. The IFAT on infected red blood cells was as described by McBride (1985).

Results

All 5 monoclonal antibodies, produced by immunizing mice with frozen sporozoites, induced a strong CSP reaction (Table 1). The CS proteins were shed very quickly at 20° C so that already after a few minutes a clear precipitate could be seen at one end of the parasite (Fig. 1). In the suspension IFAT the sporozoites were found to produce long trails (Fig. 2). The monoclonal antibodies were stage specific and showed no crossreactivity with infected red blood cells (Table 1). By using an ELISA with the synthetic peptide $(NANP)_3$ as

Table 1. Summary of the properties of the 5 monoclonal antibodies directed against the $(NANP)_3$ epitope of *P. falciparum* sporozoites

Antibodies	IFAT/ sporozoites	IFAT/ blood stages	CSP reaction	ELISA/ $(NANP)_3$	Ig classes
sp_3/B_4	1/30,000	–	+++	1/1,000,000	Ig G_{2a}
sp_3/H_3	1/25,000	–	+++	1/5,000	Ig G_1
sp_3/E_6	1/50,000	–	+++	1/170	Ig G_{2b}
sp_3/C_6	1/10,000	–	+++	1/500	Ig G_{2b}
sp_3/E_9	1/200,000	–	+++	1/100,000	Ig G_1



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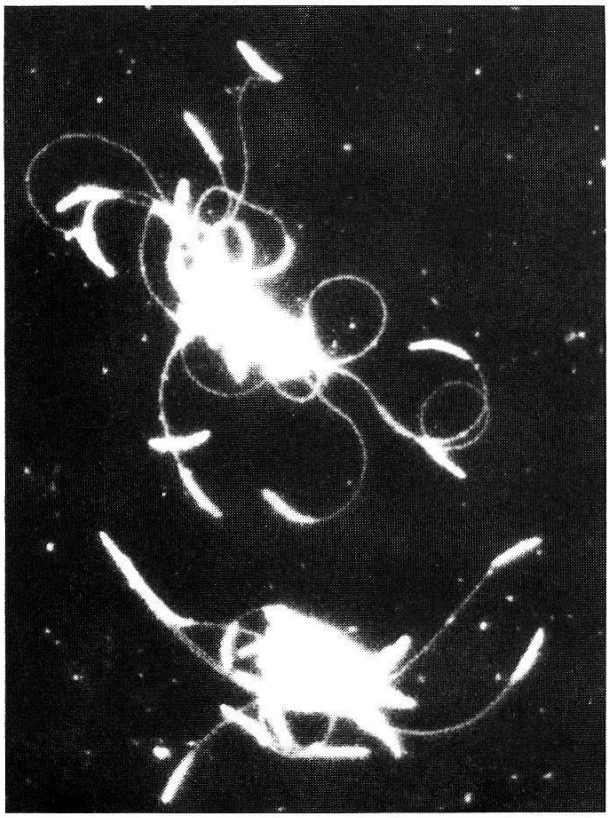

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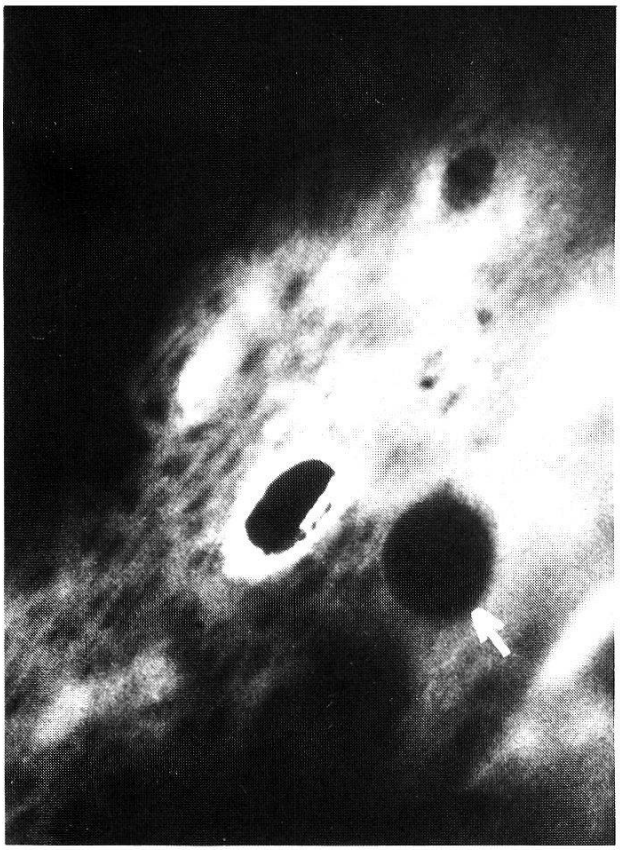
Fig. 1. Circumsporozoite protein precipitation of *P. falciparum* midgut-sporozoite in suspension, isolated 11 days after infection and incubated with the monoclonal antibody sp_3/B_4. Arrows indicate the length of the precipitation. Phase contrast, ×4330.

Fig. 2. Circumsporozoite protein precipitation of salivary gland sporozoites of *P. falciparum* after incubation with the monoclonal antibody in a suspension-IFAT. Some of the sporozoites are found in clumps, some are connected by fine fluorescent trails of circumsporozoite proteins. IFAT, ×2355.

antigen, it could be shown that the antisera had differing affinities towards this epitope. Sp_3/E_6 had the lowest affinity with a titer of 1/170 and the clone sp_3/B_4 the highest with a titer of 1/1,000,000. The clones produced antibodies of different subclasses of the immunoglobulin G class. For the present study, the monoclonal antibody sp_3/B_4 was used.



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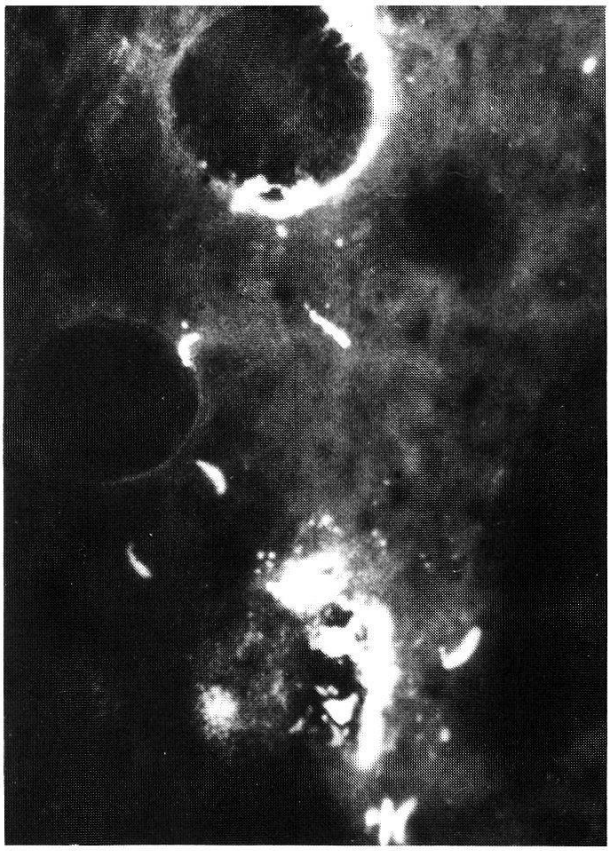

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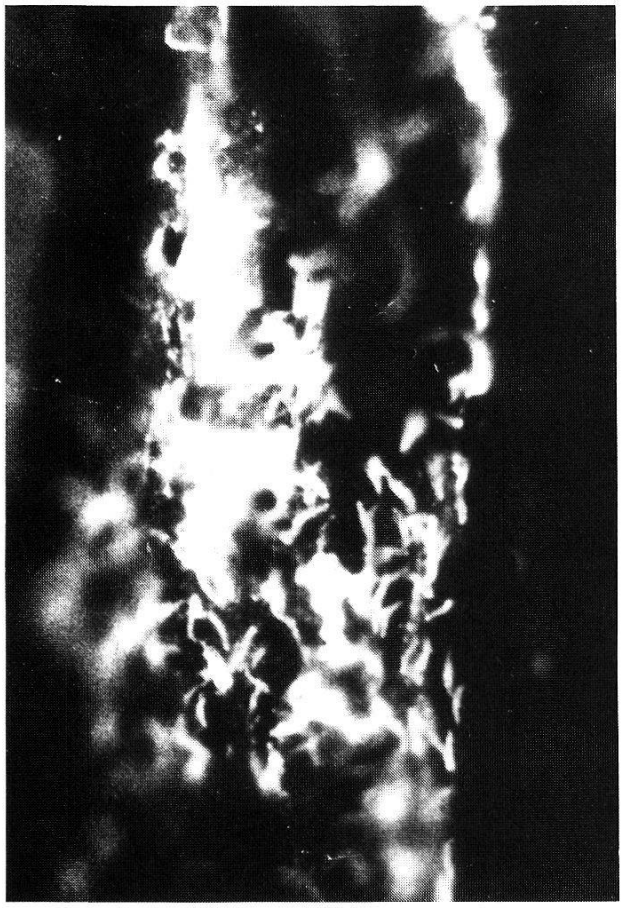

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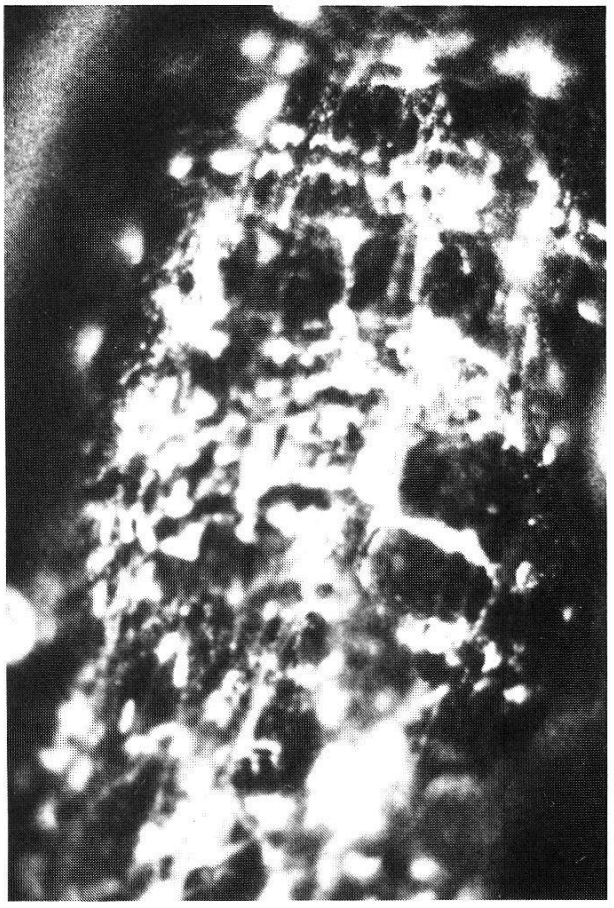

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Fig 3. Immunoreactivity of a midgut oocyst of *P. falciparum* dissected 7 days after infection. Arrow indicates an oocyst devoid of any fluorescence. IFAT, ×925.

Fig. 4. Immunoreactivity on an oocyst and midgut sporozoites of *P. falciparum* 10 days after infection. No sporozoites are found to be labelled inside the oocysts. IFAT, ×950.

Fig. 5. Immunoreactivity of large numbers of sporozoites released from mature oocysts on a midgut, 10 days after infection. IFAT, ×1250.

Fig. 6. Immunoreactivity of the midgut wall of *Anopheles stephensi* with the $(NANP)_3$ specific monoclonal antibody, 13 days after infection. IFAT, ×1125.

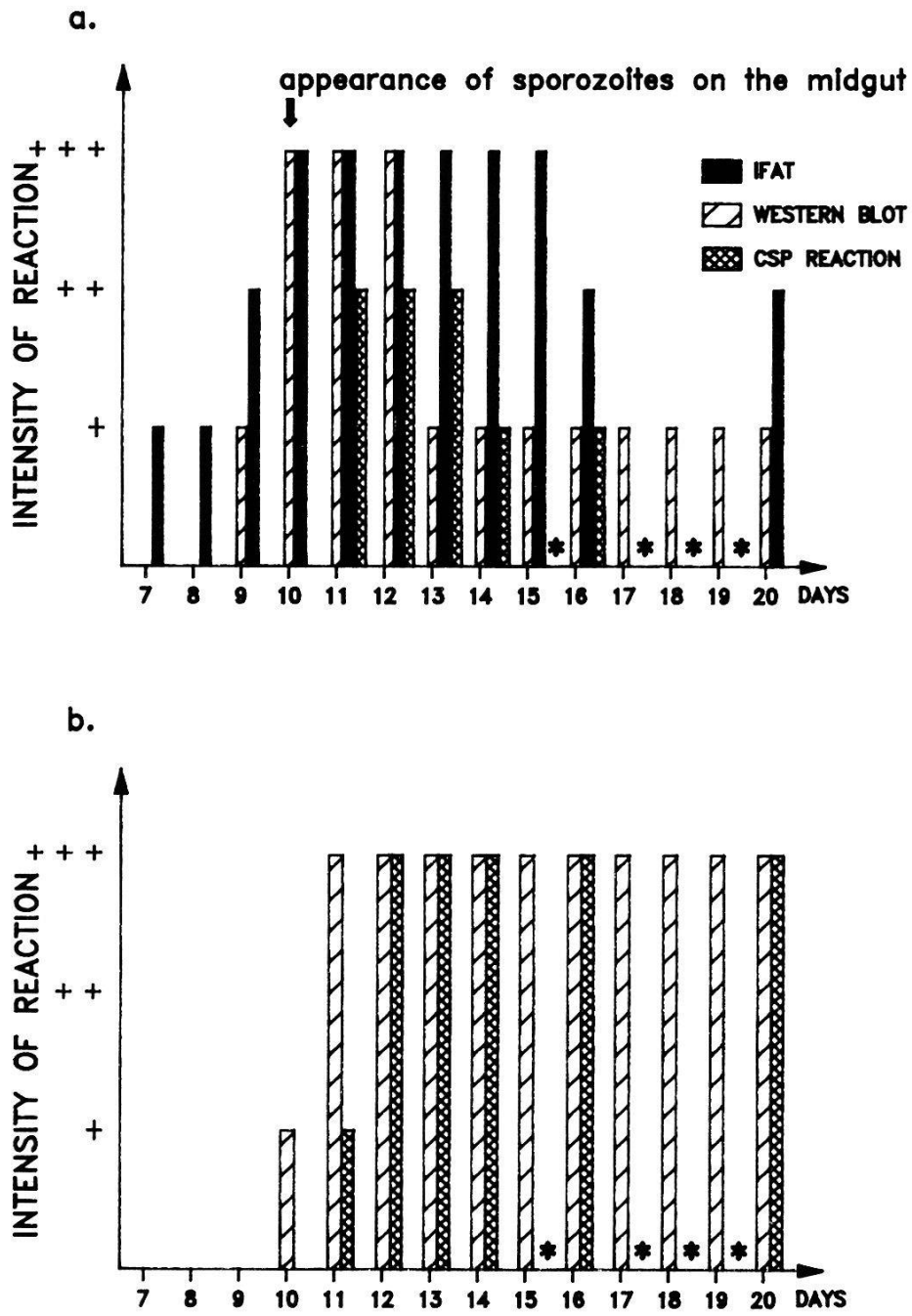

Fig. 7. Summary of the kinetics of the circumsporozoite protein development of *P. falciparum* in *Anopheles stephensi*. Immunofluorescence, Western blot analysis and CSP reaction in midgut preparations (a) and in salivary gland preparations (b). The criteria used for this figure are summarized in Table 2. CSP reactions could not be performed on midgut preparations older than 20 days owing to the lack of sufficient sporozoites. The positive immunofluorescence on midguts 20 days after infection indicates the reactivity of the midgut structures.* No data.

The appearance of the circumsporozoite protein was followed by using the IFAT procedure on dissected midguts with *P. falciparum* oocysts at different time points after infection (Figs. 3, 4, 5, 6). The monoclonal antibody was already able to detect a $(NANP)_3$ specific epitope on some oocyst walls 7 days after infection (Fig. 3) and approximately 3 days before the appearance of sporozoites. The reaction seemed to be restricted especially to some peripheral

Table 2. Summary of the parameters used to evaluate the formation of the circumsporozoite protein of *P. falciparum*

	IFAT*	Western Blot	CSP reaction
+	Some midguts have fluorescent oocysts	Not all of the three bands of the CS protein can be detected	Just a few sporozoites showed the CSP reaction
++	Many oocysts are fluorescent in every midgut. On day 20, it indicates midgut-wall fluorescence		A high number of sporozoites undergo the CSP reaction and the size of the precipitation varies from one sporozoite to another
+++	Free fluorescent sporozoites and midgut wall strongly fluorescent	All three bands are strongly stained	Practically all sporozoites show a strong CSP reaction

* Six midguts were examined each day in each of 3 experiments

parts of the oocysts. The first sporozoites were detected by the monoclonal antibody 10 days after infection, in the neighbourhood of the still-fluorescing oocysts (Figs. 4, 5). Their number increased with increasing age. Twelve to 13 days after infection the monoclonal antibody also seemed to detect epitopes outside the oocyst walls on the midgut itself (Fig. 6).

Sporozoites could be isolated from midguts 11 days after infection. Some of them showed a strong CSP reaction (Fig. 1). The number of sporozoites isolated from midguts started to decrease 14 days after infection (Fig. 7a), so that after 20 days it was not possible to collect enough sporozoites and to induce the CSP reaction. Never all sporozoites isolated from the midgut showed the precipitation reaction (data not shown).

Sporozoites could be detected and isolated from salivary glands on day 11 after infection. The sporozoites showed a strong CSP reaction (Fig. 7b) up to 20 days post infection which was the last time point assayed. The intensity of the reaction was the same whether sporozoites from the midgut or from the salivary glands were used.

The appearance of the circumsporozoite protein as detected by the IFAT- and CSP-analysis was also followed by a Western blot analysis of the proteins of infected midguts. A weak reactivity was first detected in the midgut at day 9 (Fig. 8a). At this time no reactivity could be detected in salivary gland preparations (Fig. 8b). The intensity of the reaction in midgut preparations was highest from days 10 to 12 after the infective blood meal (Fig. 8a). The antibody recognized a polypeptide of 52.5 kD, presumably the CS protein. In addition to this protein, 2 polypeptides with molecular weights of 55 and 57.5 kD were detected. The proportions of the 3 polypeptides varied with the time point of analysis, but could be recognized on midgut preparations up to 20 days post

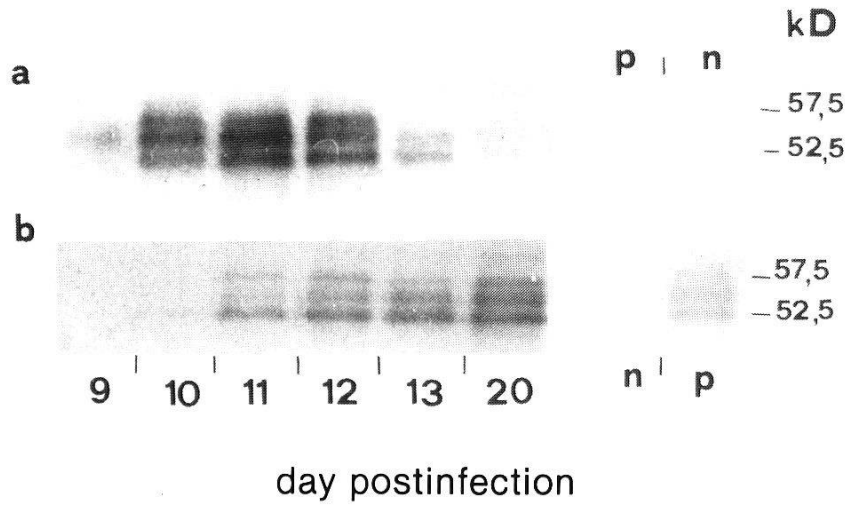

Fig. 8. Western blot analysis of *P. falciparum* infected midguts (a) and salivary glands (b) at different times (days) after infection. For each day the midguts and the salivary glands were dissected from the same 5 mosquitoes. Protein extracts of whole mosquitoes infected with *P. falciparum* (=p) and of uninfected mosquitoes (=n) served as controls. kD = kilodaltons.

infection. All 3 polypeptides could be detected in the salivary glands up to 31 days post infection (data not shown). The reactions could be inhibited by incubating the blot with the monoclonal antibody in the presence of the synthetic peptide $(NANP)_3$.

Discussion

The 5 anti-$(NANP)_3$ monoclonal antibodies described in the present paper possessed similar properties to those already described (Wirtz et al., 1987). They showed a high reactivity with the repetitive epitope $(NANP)_3$ and they were highly species-specific (data not shown).

The monoclonal antibody sp_3/B_4 was able to induce a similar CSP reaction whether the sporozoites were derived from the midgut or from the salivary glands. Several authors (Shute, 1943; Ball and Chao, 1961; Schneider, 1968; Vanderberg, 1975; Daher and Krettli, 1980) reported that midgut-sporozoites behaved similarly to salivary gland sporozoites. The sporozoites from the midgut seem to be more heterogeneous in that not all of them could be induced to produce a CSP reaction. The presence of 2 populations of sporozoites could be explained by an asynchronous maturation as described e.g. in *P. agamae,* a saurian malaria (Boulard et al., 1983) and in *P. gallinaceum* (Laurente and Krettli, 1986). It is also possible that 2 different populations of sporozoites are produced: one able to undergo a CSP reaction in the presence of a specific antibody whereas the other always lacks this capacity due to some unknown deficiencies.

The detection of the CS epitope on the oocysts as early as 7 days post infection by using the IFAT correlates well with the observations of the appearance of the CS epitope in undifferentiated oocysts using immunocytochemical techniques (Aikawa et al., 1981; Hamilton et al., 1987; Nagasawa et al., 1987; Posthuma et al., 1987). In the IFAT, the monoclonal antibody bound to peripheral areas of the oocysts, so that sporozoites within oocysts were never found to be labelled. This is in contrast to the findings of Hamilton et al. (1987a, b) and Posthuma et al. (1987) where the CS protein immunoreactivity was present on the plasmalemma and its invaginations, as well as the membrane of the sporozoites. The resolution of the IFAT did not allow the identification of the reacting structure, but it seems possible that it represents the fuzzy material beneath the oocyst wall (Sinden and Strong, 1978), which was found to be labelled (Posthuma et al., 1987). The lack of fluorescence on sporozoites inside the oocysts is explained by a possible inability of the antibodies to penetrate deeper into the oocysts.

With the beginning of the release of sporozoites, a specific binding of the monoclonal antibody to structural elements of the midgut was found. It is not clear whether this labelling is caused by secretory/excretory material present in the oocysts and released through the ruptures caused by the sporozoites. Alternatively, the released sporozoites might migrate along the midgut leaving trails of CS proteins, which adhere firmly to some midgut structures (Stewart and Vanderberg, 1987).

The Western blot technique using a peroxidase-coupled second antibody detected an $(NANP)_3$-specific epitope only 9 days after infection, 2 days later than the IFAT. It is not known whether the reduced sensitivity is caused by a possible denaturation of the CS protein epitopes, or by a lower detection sensitivity of the assay system. Positive reactions could be detected on midgut preparations up to 29 days after infection (data not shown). This prolonged reactivity could have been due to a late sporozoite maturation or to the absorbed material on the midgut, seen in the IFAT. The latter seems to be more plausible, since it was impossible to detect sporozoites from midguts dissected later than day 20 after infection. In addition, it was difficult to detect any oocysts on older midguts.

The 3 peptide-bands detected by the monoclonal antibody had somewhat lower molecular weights (52.5; 55; 57.5 kD) than published ones (58; 65; 67 kD) (Cochrane et al., 1984; Santoro et al., 1983). The differences might possibly have been caused by differing polyacrylamide concentrations and different running conditions (Vermeulen, 1983).

The appearance of the circumsporozoite protein in an early stage of oocyst maturation was surprising. It is difficult to imagine that the CS protein is synthesized at this early stage for the sole purpose of enabling the sporozoites to invade the hepatocytes of the host. The CS protein might represent a protective coat which enables the sporozoites to reach the salivary glands.

Acknowledgments

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