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Plasmodium vivax: A Monoclonal Antibody Recognizes a Circumsporozoite Protein Precursor on the Sporozoite Surface

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Gonzalez-Ceron, L., Rodriguez, M. H., Wirtz, R. A., Sina, B. J., Palomeque, O. L., Nettel, J. A., and Tsutsumi, V. 1998. *Plasmodium vivax:* A monoclonal antibody recognizes a circumsporozoite protein precursor on the sporozoite surface. *Experimental Parasitology* **90**, 203–211. The major surface circumsporozoite (CS) proteins are known to play a role in malaria sporozoite development and invasion of invertebrate and vertebrate host cells. *Plasmodium vivax* CS protein processing during mosquito midgut oocyst and salivary gland sporozoite development was studied using monoclonal antibodies which recognize different CS protein epitopes. Monoclonal antibodies which react with the CS amino acid repeat sequences by ELISA recognized a 50-kDa precursor protein in immature oocyst and additional 47- and 42-kDa proteins in older oocysts. A 42-kDa CS protein was detected after initial sporozoite invasion of mosquito salivary glands and an additional 50-kDa precursor CS protein observed later in infected salivary glands. These data confirm previous results with other *Plasmodium* species, in which more CS protein precursors were detected in oocysts than in salivary gland sporozoites. A monoclonal antibody (PvPCS) was characterized which reacts with an epitope found only in the 50-kDa precursor CS protein. PvPCS reacted with all *P. vivax* sporozoite strains tested by indirect immunofluorescent assay, homogeneously staining the sporozoite periphery with much lower intensity than that produced by anti-CS repeat antibodies. Immunoelectron microscopy using PvPCS showed that the CS protein precursor was associated with peripheral cytoplasmic vacuoles and membranes of sporoblast and budding sporozoites in development oocysts. In salivary gland sporozoites, the CS protein precursor was primarily associated with micronemes and sporozoite membranes. Our results suggest that the 50-kDa CS protein precursor is synthesized intracellularly and secreted on the membrane surface, where it is proteolytically processed to form the 42-kDa mature CS protein. These data indicate that differences in CS protein processing in oocyst and salivary gland sporozoites development may occur. © 1998 Academic Press

Index Descriptors and Abbreviations: Apicomplexa; *Plasmodium vivax; Anopheles albimanus;* sporozoite; oocyst; midgut; salivary gland; circumsporozoite protein; malaria; BSA, bovine serum albumin; CS, circumsporozoite; ELISA, enzyme-linked immunoabsoebent assay; MAb, monoclonal antibody; kDa, kilodaltons; *P. vivax, Plasmodium vivax*; PvCS1 and PvCS2, monoclonal antibodies against the *P. vivax*-210 CS repeats; PvPCS, monoclonal antibody against a nonrepetitive *P. vivax* CS epitope.

INTRODUCTION

Circumsporozoite (CS) proteins of *Plasmodium* parasites are important antimalarial vaccine targets because of their location on the surface of infective sporozoites inoculated during *Anopheles* mosquito biting and their immunogenicity (Good *et al.* 1988; Stoute *et al.* 1997; Tapchaisri *et al.* 1983; Vermeulen *et al.* 1982). CS proteins of all Plasmodia species contain a central region, variable in length, with species-specific repeated amino acid sequences. Repeat regions vary little among isolates of the same species, except for *Plasmodium vivax*, in which two different sequences (CS 210 and

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CS 247) have been identified (Arnot *et al.* 1985; Rosenberg *et al.* 1989).

CS proteins are expressed in undifferentiated mosquito midgut oocysts (Meis *et al.* 1992; Posthuma *et al.* 1988) and cover the surface of fully formed sporozoites in mature oocysts and salivary glands (Aikawa *et al.* 1981; Fine *et al.* 1984). The intracellular location of the CS precursor and mature protein in salivary gland sporozoites was inferred by a combination of surface iodination, pulse–chase metabolic labeling, and selective elimination of the mature protein using partial trypsin digestion of intact sporozoites isolated from salivary glands (Fine *et al.* 1984; Yoshida *et al.* 1981). Pulse–chase experiments followed by immunoprecipitation using an anti-CS repeat monoclonal antibody (MAb) suggested that two high molecular weight CS proteins are synthesized and then processed to form one lower molecular weight mature CS protein (Cochrane *et al.* 1982; Ozaki *et al.* 1983; Yoshida *et al.* 1981). A lack of detectable surface iodination and trypsin sensitivity indicated that CS protein precursors were located intracellularly (Posthuma *et al.* 1988) and that only the mature protein is exposed on the sporozoite surface. However, these experiments were conducted using sporozoites isolated from salivary glands and no immunological reagents specific to CS protein precursor epitopes were available to establish the precise location of the precursor proteins.

In this study, we investigated the production and location of *P. vivax* CS proteins in oocyst and salivary gland sporozoites using three MAbs that recognize different epitopes of the precursor and mature *P. vivax* CS proteins. Our results indicate that CS protein precursors are more numerous in oocyst sporozoites than in sporozoites located in salivary glands, confirming previous reports in other *Plasmodium* species (Boulanger *et al.* 1995), and that these precursors are synthesized intracellularly, secreted on the parasite surface, and subsequently processed to the mature CS protein. CS protein processing mechanisms appear to differ in oocyst and salivary gland sporozoites.

MATERIALS AND METHODS

Parasites and mosquitoes. *P. vivax* malaria-infected subjects diagnosed by Giemsa-stained blood smears at the Center for Malaria Research in Tapachula, Chiapas, Mexico, provided 10-ml blood samples in heparin-treated tubes, after an informed consent form was signed. Patients were immediately treated with chloroquine/primaquine (World Health Organization 1967).

A strain of *Anopheles albimanus* female mosquitoes (white stripped phenotype) very susceptible to *P. vivax* infection (Chan *et al.* 1994) (2–6 days postemergence) were fed on *P. vivax*-infected blood (Ramsey *et al.* 1994), through parafilm membranes. Engorged mosquitoes were maintained with 10% sucrose *ad libitum* at 23–28°C, 70–80% relative humidity. All mosquitoes fed with one patient's blood were considered one lot. To estimate the infection rate, 10 mosquitoes from each lot were dissected on Day 7 postinfective blood meal, midguts were stained with 1% mercurochrome (Eyles 1950), and oocyst numbers recorded after viewing with a light microscope (magnification 400×). Salivary gland infection rates were determined by dissection and sporozoite numbers were counted in a hemocytometer. The *P. vivax* CS protein variant type was determined by IFA using a anti-repeat-specific MAb (PvCS247, Wirtz *et al.*, unpublished).

Monoclonal antibodies. Hybridomas secreting MAbs against the CS protein of *P. vivax* were prepared as described previously (Wirtz *et al.* 1985). Three MAbs were produced from BALB/c-immunized mice: MAbs PvCS1 (IgG1) and PvCS2 (IgM) were produced from mice immunized by biting of sporozoite-infected mosquitoes and MAb PvVCS (IgM) was obtained by immunization with isolated salivary gland sporozoites (Wirtz *et al.* 1992).

Immunoblotting. Both *P. vivax* 210-infected midguts and salivary glands of 10 mosquitoes were dissected on Days 5 to 12 postinfection in PBS containing protease inhibitors [25 μg/ml TLCK (*N-p*-tosyl-L-lysine chloromethyl ketone), 50 μg/ml TPCK (*N*-tosyl-L-phenylalanine chloromethyl ketone), 348 μg/ml PMSF (phenylmethylsulfonyl fluoride), 50 μ/ml chymostatin, 50 μg/ml leupeptin, 1 mg/ml aprotinin, 2 mM EDTA (ethylenediaminetetraacetic acid)] (Sigma Chem. Co., St. Louis, MO) and stored at −70°C. Oocyst-infected midguts and salivary gland sporozoites were dissolved in sample buffer, electrophoresed in 10% SDS–polyacrylamide gels (SDS–PAGE) (Laemmli 1970), along with broad range molecular weight markers (Sigma Chem. Co.), and electroblotted onto nitrocellulose membranes. Blots were incubated with, PvCS1 or PvCS2 (1/10 tissue culture supernatant) and PvPCS (ascites fluid 1:300), followed by horseradish peroxidase-conjugated goat anti-mouse IgG+M (Pierce Chem. Co., Rockford, IL). Antibody binding was detected with chemiluminiscense reagents (ECL, Amersham, Buckinghamshire, UK) and exposure to X-Omat AR film (Eastman Kodak Co. Hemel Hempstead, Herts, UK) for 10–60 s. Uninfected midguts or salivary gland preparations were included as controls.

For two-dimensional electrophoresis, *P. vivax*-infected salivary glands placed in sample buffer (9.5 M urea, 2% Triton X-100, 5% mercaptoethanol, and 1.6 and 0.4% ampholine, pH range 5–8 and 3–10 (Bio–Rad Lab., Richmond, CA), were isoelectrofocused, followed by separation by 10% SDS–PAGE under reducing conditions, and electrotransferred to nitrocellulose membranes. After treatment with PvPCS and chemiluminescent detection, the membrane was washed, reincubated with PvCS2, and reexposed.

ELISA. All MAbs were tested in an ELISA (Wirtz *et al.* 1990) using bovine serum albumin (BSA)-conjugated peptides $(GRDRADGQPA)_3$ as *P. vivax*-210 strain CS repeat region antigen (Arnot *et al.* 1985) and $(ANGAGNQPG)_2$ as *P. vivax*-247 strain CS repeat region antigen (Rosenberg *et al.* 1989) as capture antigen.

Immunofluorescence assays. MAbs were tested by IFA (Nardin *et al.* 1982) using *P. vivax* 210 strain air-dried sporozoites obtained from mosquitoes infected with blood from 20 Mexican and four Thai patients and *P. vivax* 247 strain sporozoites obtained in the same manner from two Mexican and two Thai patients. MAbs were also tested against sporozoites of *P. falciparum* (NF54 strain); *P. knowlesi* (H); *P. yoelli*

(17XNL); *P. berghei* (ANKA); and *P. gallinaceum* (8A). MAbs PF-2A10 (anti-*P. falciparum* CS repeat region) (Zavala *et al.* 1985), PB-36 (anti-*P. bergehi* CS repeat region) (Sina *et al.* 1995), Py1B1 (anti-*P. yoelii* CS repeat region) (Wirtz *et al.*, unpuglished), and Pg/Pk-6G1 (anti-*P. vivax* sporozoite, which cross-reacts with *P. gallinaceum* and *P. knowlesi* sporozoites) (Wirtz *et al.*, unpublished), were used as positive controls. PvPCS and PvCS2 were also tested by IFA using fresh live *P. vivax* 210 sporozoites. An unrelated mouse IgM Mab which reacts with the basal lamina of *An. albimanus* salivary glands (Gonzalez-Ceron *et al.*, unpublished) was used as a negative control.

Immunogold electron microscopy. *Plasmodium vivax* 210 oocyst-infected midguts and salivary gland sporozoites were fixed, embedded in LR white resin, and thin sections were prepared as described previously (Aikawa and Atkinson 1990). Grids with tissue samples were incubated with PvPCS (mouse ascites) and PvCS2 (culture supernatant), followed by goat anti-mouse IgM labeled with 1-nm gold particles (Aikawa and Atkinson 1990), and examined with a Zeiss EM-10 transmission electron microscope. Control preparations were treated with a normal mouse ascites fluid (induced by injection of SP_2/O hybridoma cells into the peritoneal cavity of pristane-treated mice), tissue culture medium (RPMI with 20% fetal calf serum), or an unrelated mouse IgM MAb which reacts specifically with the basal lamina of mosquito salivary glands (Gonzalez-Ceron *et al.*, unpublished).

RESULTS

PvCS and PvPCS antibody specificity. Monoclonal antibodies derived from *P. vivax* sporozoite-immunized mice were characterized by sporozoite IFAs and ELISAs using BSA-conjugated CS repeat peptide antigen. In IFAs, PvPCS stained both *P. vivax* 210 (Fig. 1A) and 247 dried sporozoites (data not shown), producing intense fluorescent spots on the periphery and at both ends of the parasite. This reaction was less intense than that produced by PvCS1 and PvCS2. These antibodies produced intense homogeneous staining of the periphery of *P. vivax* 210 sporozoites (Fig. 1B), but did not react with *P. vivax* 247 sporozoites. When tested with live *P. vivax* 210 sporozoites, PvPCS and PvCS2 produced homogeneous surface staining of the parasite, but the intensity of the reaction was weaker for PvPCS (Fig. 1C) compared to PvCS2 (Fig. 1D). PvPCS reacted with all *P. vivax* 210 and 247 Mexican and Thai sporozoites tested. A very weak reaction was observed with *P. falciparum* sporozoites by IFA, but no reaction was observed with the other *Plasmodium* species tested. PvCS1 and PvCS2 reacted in an ELISA with the *P. vivax* 210 CS repeat region (data not shown). In contrast, PvPCS did not react with either *P. vivax* CS 210 or 247 repeat peptides (data not shown). These results suggested that PvCS1 and PvCS2 recognized *P. vivax* strain-specific CS repeat epitopes and PvPCS recognized a nonrepeat CS epitope conserved among all the *P. vivax* strains tested.

MAb PvPCS reacted with the CS protein precursor. On blots of SDS gels, PvPCS appeared to react only with a 50-kDa band of 8-day oocysts (Fig. 2) and in salivary gland *P. vivax* 210 sporozoite (Fig. 2) preparations. PvPCS also reacted with a 61-kDa band in *P. vivax* 247 sporozoite preparations, while PvC247 recognized two bands (61 and 48 kDa) in Western blots (data not shown). To determine whether PvPCS and PvCS2 recognize the same protein, immunoblots prepared from salivary gland *P. vivax* 210 sporozoite samples run in two-dimensional gel electrophoresis were treated sequentially with these antibodies. PvPCS recognized a single spot of 50 kDa on the acidic side of the blot (Fig. 3A) and

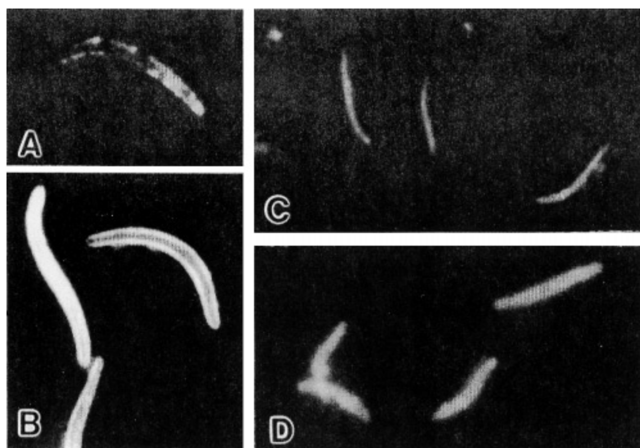

FIG. 1. Indirect immunofluorescence of *P. vivax* 210 sporozoites (Mexican strains). Air-dried (A and B) and live (C and D) sporozoites were treated with PvPCS (A and C) and PvCS 2 (B and D).

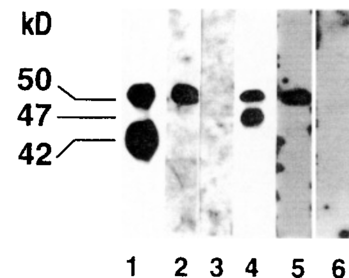

FIG. 2. Immunoblot analysis of *P. vivax* 210 oocyst and sporozoite protein extracts. Samples were run in a 8% polyacrylamide gel and blotted to nitrocellulose membranes. Lanes 1 and 2 contain salivary gland sporozoites; lane 3, uninfected salivary glands; lanes 4 and 5, 8-day postfeeding oocyst-infected midguts; lane 6, uninfected midguts (lane 5 has 12 times more total protein than lane 4). Lanes 1 and 4 were incubated with PvCS2 and lanes 2, 3, 5, and 6 were incubated with PvPCS.

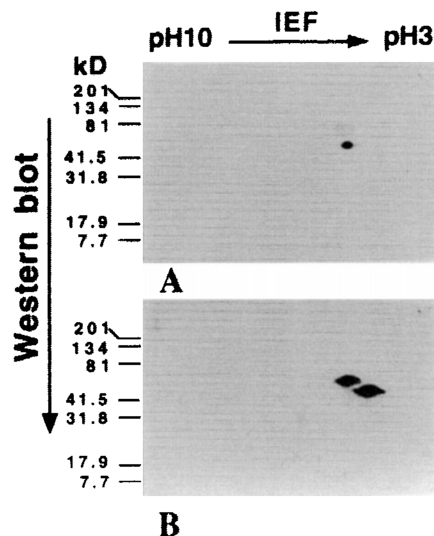

FIG. 3. Immunoblot of two-dimensional gel electrophoresis of *P. vivax* 210 salivary gland sporozoite protein extracts. Protein extracts equivalent to 5×10^4 sporozoites run in a two-dimensional electrophoresis gel and electroblotted to nitrocellulose filter. After incubation with PvPCS, the membrane was treated with ECL reagents and autoradiographed (A). Subsequently, the same blot was washed in PBS and reincubated with PvCS2 (B).

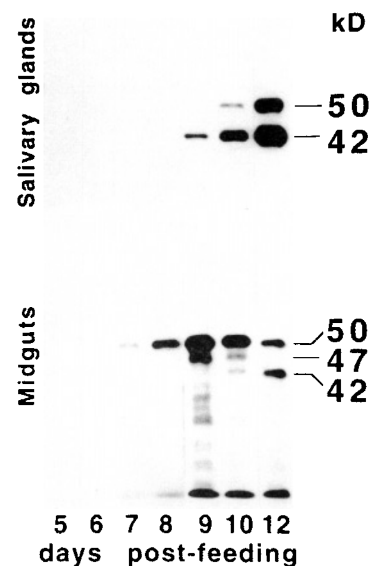

FIG. 4. Immunoblot analysis of CS protein expression in *P. vivax* 210-infected *Anopheles albimanus* mosquitoes. *P. vivax*-infected midguts and salivary gland sporozoite extracts were prepared on Days 5 to 12 after infection from the same mosquito lot. Samples were run in a 10% polyacrylamide gel and blotted to nitrocellulose membranes. Membranes were treated with PvCS1.

PvCS2 recognized the same spot and another more acidic 42-kDa spot (Fig. 3B). These results confirm that PvPCS reacts with a conserved epitope found only in the high molecular weight CS precursor protein of both *P. vivax* variants.

Kinetics of P. vivax *CS protein expression.* The kinetics of *P. vivax* CS protein expression were determined by analyzing immunoblots of oocyst and salivary gland sporozoites samples, obtained from mosquitoes at different times after infection, treated with PvCS1 (Fig. 4). Detection of CS protein in oocyst-infected midguts varied among different mosquito infections. In an infection having an average number of 30 oocysts/midgut, a 50-kDa band was observed up to 14 days postinfection. Between Days 7 and 14, the intensity of the band of 47 kDa decreased, while the intensity of the 42-kDa band increased (Fig. 4). A 47-kDa band was clearly visible in all mosquito lots analyzed ($n = 16$), but in some cases, smearing between the 50- and the 42-kDa bands made the observation of the 47-kDa band difficult.

Plasmodium vivax sporozoites first appeared in salivary glands between Days 9 and 12 postinfection. Unlike infected midguts, only two bands (50/42 kDa) were consistently detected by PvCS1 in salivary gland sporozoite samples. The 42-kDa band was first detected on Day 9 and both bands were observed on Days 10–14 (Fig. 4). PvCS2 also recognized the 42- and 50-kDa bands in *P. vivax* salivary gland sporozoites (Fig. 2) and was used in further experiments for comparison with the same isotype MAb as PvPCS.

The 50-kDa CS protein precursor is located on the sporozoite surface. Immunoelectron microscopy analysis was used to determine the location of CS proteins recognized by PvPCS in developing oocyst and salivary gland sporozoites. PvPCS labeled the plasmalemma of compact oocysts (Day 6) very sparsely (data not shown). Gold particles heavily labeled cytoplasmatic as well as peripheral vacuoles which appeared in oocysts on Days 7–8 (Fig. 5A). Heavily labeled electron-dense areas were observed in cytoplasm of sporoblasts. Gold particles were also associated with sporoblast membranes (on Days 9–10); this was also observed along the reticulum and membranous areas. In preparations showing sporozoite budding (on Days 11–12), gold particles were observed in a patchy pattern on the membranes of the sporozoites (Fig. 5B). In fully formed sporozoites some rhoptry secretory organelles were also labeled (Fig. 5C).

In sporozoites in salivary glands, PvPCS bound to intracellular organelles. Gold particles intensively labeled micronemes in most of the sporozoites observed (Fig. 5D). Rhoptry labeling varied among different sporozoites. PvPCS also labeled the inner and the outer sporozoite membranes (Figs. 5D and 5E). PvCS2 labeled sporozoite membranes in a distribution similar to that of PvPCS, but more intensively

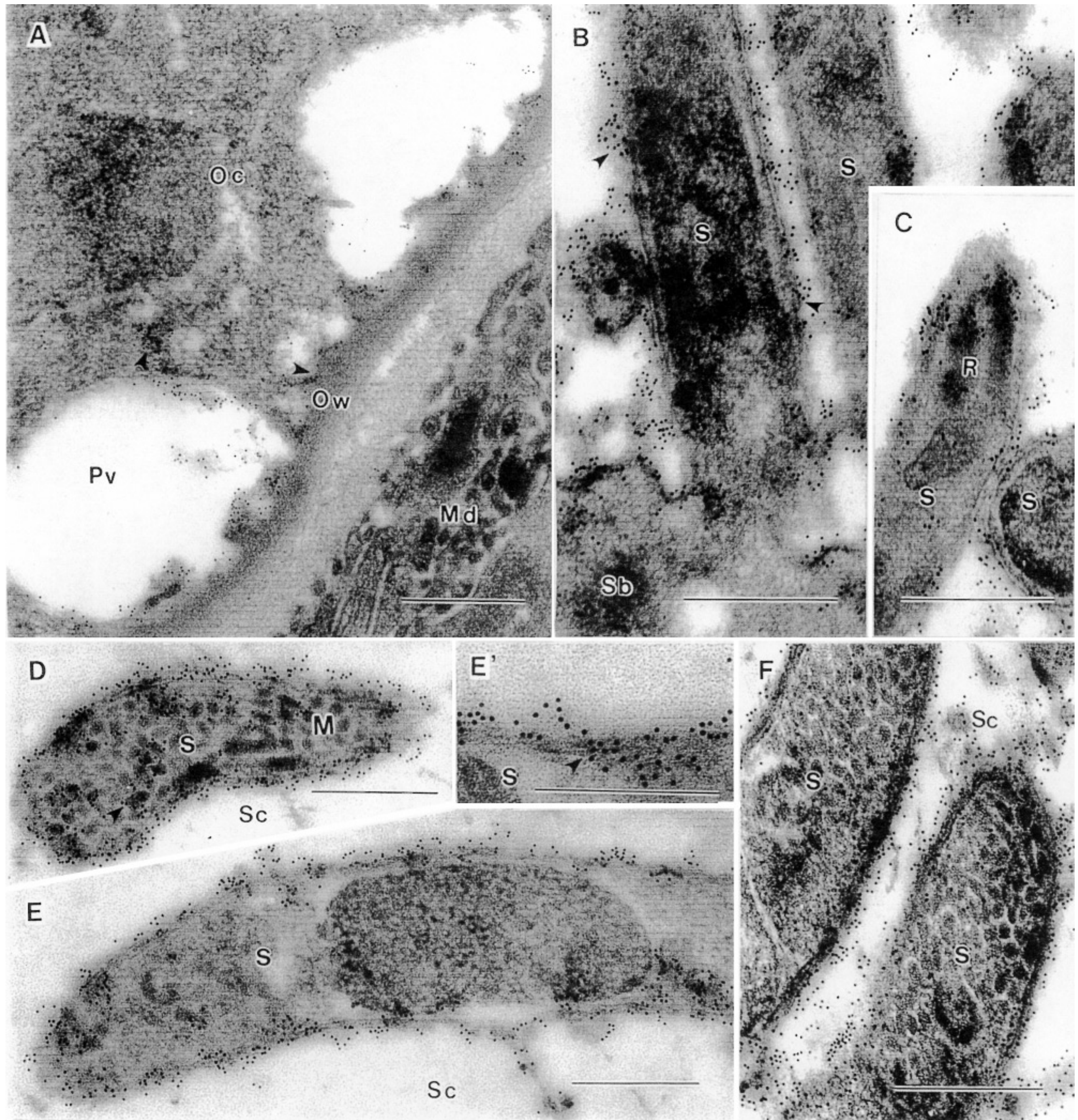

FIG. 5. Immunogold electron microscopy of sections of *P. vivax* developing oocysts and sporozoites treated with PvPCS (A–E) and PvCS2 (F): (A) Peripheral vacuoles developing oocyst; (B) sporoblast showing budding sporozoites; (C) oocyst sporozoites; (D–F) salivary gland sporozoites. Oc, oocyst; Ow, oocyst wall; Pv, peripherial vacuoles; Mc, midgut cell; Sc, secretory cavity; S, sporozoite; Sb, sporoblast; M, micronemes; R, rhoptries. Bars indicate 0.5 μm.

(Fig. 5F). These observations confirmed that the CS precursor protein is secreted onto the sporozoite surface.

DISCUSSION

The CS protein comprises the most abundant molecule on the surface of mature salivary gland sporozoites (reviewed in Nussenzweig and Nussenzweig 1985) and sporozoites released from mosquito midgut oocyst (Hamilton *et al.*, 1987; Nagasawa *et al.* 1987, 1988). This protein participates in the hepatocyte recognition and invasion by the parasite (Cerami *et al.* 1992; Frevert *et al.* 1993) and may play a role in the invasion of the mosquito salivary gland by sporozoites (Warburg *et al.* 1992). However, some differences between oocyst and salivary gland sporozoites have been detected (Sinden and Garnham 1973; Sterling *et al.* 1973; Turner 1981). Sporozoites recovered from salivary glands loose their capacity to reinvade this organ (Touray *et al.* 1992) and their capacity to invade hepatocytes can be up to 10,000 greater than that of pre-salivary gland parasites, indicating that development of infectivity occurs in the salivary gland (Vanderberg 1975).

Previous studies on *Plasmodium* CS protein expression were conducted using MAbs that recognized amino acid repeat sequences that form the central region of the mature protein (Cochrane *et al.* 1982; Hamilton *et al.* 1987; Meis *et al.* 1992; Nagasawa *et al.* 1987; Nardin *et al.* 1982, Yoshida *et al.* 1981). These studies consistently identified two or three CS proteins in salivary gland sporozoites. Pulse–chase experiments suggested that the larger CS proteins were precursors of the mature smaller protein. In our study, we compared binding of a MAb (PvCS2), which recognized the repeat portion of the *P. vivax* CS protein and therefore bound the precursors (50 and 47 kDa) and mature (42-kDa) CS protein, and a MAb (PvPCS) which only binds the 50-dKa precursor CS protein. Double staining of sporozoite proteins separated by two-dimensional electrophoresis using PvCS2 and PvPCS demonstrated that both antibodies recognized different epitopes of the same protein and that the latter binds to a CS protein epitope which is lost from the largest precursor during maturation. The p*I* value of the 42-kDa band is reduced after the 50-kDa CS peptide is processed, which suggests a cleavage of basic amino acid residues, as has been suggested in *P. falciparum, P. knowlesi, P. berghei,* and *P. cynomolgi* (Santoro *et al.* 1983). The reactivity of PvPCS with *P. vivax* 210 and 247 sporozoites suggests that this epitope is conserved among *P. vivax* parasites.

During our studies, we observed that the initiation of CS protein synthesis in developing oocysts occurred at variable times postinfection. CS protein was first detected in a chimpanzee-adapted *P. vivax* strain (Chesson) using immunoelectron microscopy on Day 6 postinfection (Meis *et al.* 1992). Our experiments were performed with several parasite lots obtained from different patients. CS proteins were first detected in oocysts on Days 6 to 9 postinfective blood meal and the first detection of sporozoites in mosquito salivary glands varied from Days 6 to 13. Variation in temperature can influence sporogonic development and protein expression (Garnham 1964); however, differences between parasites lots were observed, even if infected mosquitoes, from the same colony generation, were maintained side by side under the same conditions, suggesting that undetermined parasite factors may influence the sporozoite maturation rate.

Immunoblot experiments demonstrated that the 42-kDa mature CS protein consistently appeared in *P. vivax* sporozoites first invading salivary glands and that the 50-kDa band was only detected on subsequent days. Similar results were reported for *P. falciparum* sporozoites infecting *An. stephensi* mosquitoes (Golenda *et al.* 1990), suggesting that CS protein synthesis stops during parasite migration to the salivary glands, but restarts after invasion. Two CS protein precursors (50 kDa, common precursor, and 47 kDa, intermediate precursor) were detected in *P. vivax* sporozoites obtained from oocysts but only the 50-kDa band was evident in salivary gland parasites. These observations confirm previous results obtained by Boulanger *et al.* (1995), who detected a ladder of one to four precursor proteins in *P. berghei* and *P. gallinaceum* oocysts that were not seen in parasites obtained from salivary glands. This may indicate that CS protein processing differs between presalivary and salivary sporozoites at the sites of cleavage. However, results obtained in experiments based on immunoprecipitation after pulse–chase labeling of *P. berghei* (Yoshida *et al.* 1981) and *P. knowlesi* (Cochrane *et al.* 1982) salivary gland sporozoites showed that the intermediate CS protein precursor was detected only during the initial chase period, suggesting that it has a much shorter life span than the larger precursor, which was detected throughout the whole chase period. Although variation among parasite species may exist, the results of these experiments should be interpreted cautiously because of possible modifications introduced during the isolation of the parasites from salivary glands.

The formation of sporozoites in *P. vivax* oocysts followed the pattern previously described in other Plasmodia (Aikawa and Sterling 1974; Sinden and Strong 1978). CS protein distribution among different oocyst and sporozoite subcellular structures was similar to that described in *P. falciparum*

(Posthuma *et al.* 1988), *P. berghei* (Nussenweig and Nussenzweig 1985), *P. ovale* (Hamilton *et al.* 1987), and *P. malariae* (Sinden and Garnham 1973). Immunoelectron microscopy using PvPCS confirmed the presence of the 50-kDa CS protein precursor on the surface of fully formed oocyst and salivary gland sporozoites. In previous metabolic labeling experiments, selective elimination of the mature molecule in trypsin-treated *P. berghei* (Yoshida *et al.* 1981) and *P. knowlesi* (Fine *et al.* 1984) sporozoites suggested that CS proteins were synthesized and processed intracellularly and only the mature protein was transported to the parasite surface. However, in these experiments, it is possible that CS protein precursors on the parasite surface were also eliminated by trypsin treatment, while intracellular precursors remained and therefore were still detected. The absence of CS protein precursors in preparations obtained by immunoprecipitation of ^{125}I-surface-labeled *P. berghei* sporozoites (Yoshida *et al.* 1981) was also taken as evidence of the absence of the precursors on the parasite surface. However, it is possible that in these experiments, inefficient metabolic labeling of the mature Cs protein sporozoites isolated from salivary glands and the small amount of CS protein precursor may have precluded their detection on the sporozoite surface.

Plasmodium sporozoites are morphologically similar to invasive stages of other Apicomplexan species possessing rhoptries and other specialized organelles at their apical end, through which membrane- or surface-associated secretory products are released (Golenda *et al.* 1990; Sam-Yellowe 1996; Stewart *et al.* 1985). In oocysts, CS proteins are embedded directly into the parasite surface during sporozoite formation. However, the presence of PvPCS labeling in rhoptries and micronemes of *P. vivax* salivary gland sporozoites indicates that at this stage, CS protein precursors are released from these organelles and translocated to the parasite surface, as previously proposed by Stewart and Vanderberg for the mature CS protein (Stewart and Vanderberg 1991). The processing mechanisms of the CS protein precursors at the parasite surface await clarification. It is possible that they involve proteolytic cleavage by surface membrane-associated proteases, as demonstrated for MSP-1 (merozoite surface protein) (Blackman *et al.* 1993) and EBA 175 (erythrocyte binding antigen) (Kain *et al.* 1993), two proteins on the surface of *P. falciparum* merozoites stepwise processed during red blood cell invasion.

Differences in CS processing between oocyst and salivary gland parasites result in the exposure of different parts of the molecule and may be indicative of a different function for the mature and intermediate forms of the protein. Two main roles have been attributed to CS proteins: motility (Stewart and Vanderberg 1991) and as a ligand in hepatocyte recognition (Touray *et al.* 1992). Vanderberg has suggested that the release of mature CS protein from the sporozoite surface results in a gliding progress of the parasite. This form of motility has been observed only in salivary gland parasites and it is still unknown if motility is necessary for the parasite to reach this organ. The gliding process bears similarities to the circumsporozoite reaction, occurring in the presence of anti-CS protein antibodies, which also requires CS protein detachment from the parasite surface. Interestingly, the circumsporozoite reaction occurs only in salivary gland parasites, but not in those released from oocysts, indicating possible differences in their CS protein conformation. In both phenomena, the relationship of CS protein detachment from the parasite and the processing of CS precursors is unknown.

Pre-salivary gland sporozoites perform poorly in hepatocyte recognition (Turner 1981) and it has been suggested that other proteins (e.g., thrombospondin related anonymous protein, TRAP) also participate in this process (Robson *et al.* 1995). On the other hand, sporozoites obtained from salivary glands have a diminished capacity to reinvade these organs (Turner 1981), indicating that a putative ligand may be lost after the initial invasion. Recently, it was demonstrated that TRAP null sporozoites are not infective for salivary glands or hepatocytes (Sultan *et al.* 1997), indicating that the expression of this protein does not explain the differential infectivity of salivary gland and oocyst sporozoites. Taking into account the presence of an intermediate CS protein precursor, which only appears before salivary gland invasion, and that antibodies against CS proteins have shown to block this organ invasion by sporozoites (Warburg *et al.* 1992), it is tempting to speculate that this precursor might function as a ligand for molecules on the surface of salivary glands.

These may not be the only functions of CS proteins. It was recently shown that disruption of the CS protein gene in *P. berghei* resulted in an arrest of oocyst development at the stage of sporozoite budding (Menard *et al.* 1997), indicating a role of CS proteins in sporozoite formation. The presence of CS protein precursors associated with membrane structures during sporozoite formation warrants further studies to elucidate whether the precursors or the mature protein participate in the oocyst maturation process and/or salivary gland invasion.

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REFERENCES

Aikawa, M., and Atkinson, C. T. 1990. Immunoelectron microscopy of parasites. *Advances in Parasitology* **29,** 151–214.

Aikawa, M., and Sterling, C. R. 1974. Ultrastructure of the major intracellular protozoa. *In* "Intracellular Parasitic Protoza" (M. Aikawa and C. R. Sterling, Eds.), pp. 24–27. Academic Press, New York.

Aikawa, M., Yoshida, N., Nussenzweig, R., and Nussenzweig, V. 1981. The protective Antigen of malarial sporozoites (*Plasmodium berghei*) is a differentiation antigen. *Journal of Immunology* **126,** 2494–2495.

Arnot, D. E., Barnwell, J. W., Tam, J. P., Nussenzweig, V., Nussenzweig, R. S., and Eneas, V. 1985. Circumsporozoite protein of *Plasmodium vivax*: Gene cloning and characterization of the immunodominant epitope. *Science* **230,** 815–818.

Blackman, M. J., Chappel, J. A., Shai, S., and Holder, A. A. 1993. A conserved parasite serine protease processes the *Plasmodium falciparum* merozoite surface protein-1. *Molecular and Biochemical Parasitology* **62,** 103–114.

Boulanger, N., Charoenvit, Y., Krettli, A., and Betschart, B. 1995. Developmental changes in the circumsporozoite proteins of *Plasmodium berghei* and *Plasmodium gallinaceum* in their mosquito vectors. *Parasitology Research* **81,** 58–65.

Cerami, C., Frevert, U., Sinnis, P., Takacs, B., Clavijo, P., Santos, M. J., and Nussenzweig, V. 1992. The basolateral domain of the hepatocyte plasma membrane bears receptors for the circumsporozoite protein of *Plasmodium falciparum* sporozoites. *Cell* **70,** 1021–1033.

Chan, A. S. T., Rodriguez, M. H., Torres, J. A., Rodriguez, M. C., and Villarreal, C. 1994. Susceptibility of three laboratory strains of *Anopheles albimanus* (Diptera: Culicidae) to coindigenous *Plasmodium vivax* in Southern Mexico. *Journal of Medical Netomology* **31,** 400–403.

Cochrane, A. H., Santoro, F., Nussenzweig, V., Gwatz, R. W., and Nussenzweig, R. S. 1982. Monoclonal antibodies identify the protective antigens of sporozoites of *Plasmodium knowlesi. Proceedings of the National Academy of Sciences USA* **79,** 5651–5655.

Eyles, D. E. 1950. A stain for malarial oocysts in temporary preparations. *Journal of Parasitology* **36,** 501.

Fine, E., Aikawa, M., Cochrane, A. H., and Nussenzweig, R. S. 1984. Immuno-electron microscopy observations on *Plasmodium knowlesi* sporozoites: Localization of protective antigen and its precursors. *American Journal of Tropical Medicine and Hygiene* **33,** 220–226.

Frevert, U., Sinnis, P., Cerami, C., Shreffer, W., Takacs, B., and Nussenzweig, V. 1993. Malaria circumsporozoite protein binds to heparan sulfate proteoglycan associated with the surface membrane of hepatocytes. *Journal of Experimental Medicine* **177,** 1287–1298.

Garnham, P. C. C. 1964. Factors influencing the development of protozoa in their arthropodan host. *In* "Host–Parasite Relationships in Invertebrate Hosts," (A. E. R. Taylor, Ed.), pp. 35–50. Symposium of the British Society for Parasitology, Vol. 2. Blackwell, Oxford.

Golenda, F. G., Starkweather, W. H., and Wirtz, R. A. 1990. The distribution of circumsporozoite protein (CS) in *Anopheles stephensi* mosquitoes infected with *Plasmodium falciparum* malaria. *Journal of Histochemistry and Cytochemistry* **38,** 475–481.

Good, M. F., Berzofsky, J. A., and Miller, L. H. 1988. The T cell response to the malaria circumsporozoite protein: An immunological approach to vaccine development. *Annual Review of Inmunology* **6,** 663–668.

Hamilton, A. J., Davies, C. S., and Sinden, R. E. 1987. Expression of circumsporozoite proteins revealed in situ in the mosquito stages of *Plasmodium berghei* by the lowicryl-immunogold technique. *Parasitology* **96,** 273–280.

Kain, K. C., Orlandi, P. A., Haynes, J. D., Sim, K. L., and Lanar, J. D. 1993. Evidence for two-stage binding by the 175-kDa erythrocyte binding antigen of *Plasmodium falciparum. Journal of Experimental Medicine* **178,** 1497–1505.

Lawmmli, E. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.

Meis, J. F. G. M., Croes, H., Mons, B., Van Belkum, A., and Ponnudurai, T. 1992. Localization of circumsporozoite protein in the sporogonic stages of *Plasmodium vivax. Parasitological Research* **78,** 165–167.

Ménard, R., Sultan, A. A., Cortex, C., Altszuler, R., Van Dijk, M. R., Janse, C. J., Waters, A. P., Nussenzweig, R. S., and Nussenzweig, V. 1997. Circumsporozoite protein is required for development of malaria sporozoites in mosquitoes. *Nature* **385,** 336–340.

Nagasawa, H., Proceli, P. M., Atkinson, C. T., Campbell, G. H., Collins, W. E., and Aikawa, M. 1987. Localization of circumsporozoite protein of *Plasmodium ovale* in midgut oocysts. *Infection and Immunity* **55,** 2928–2932.

Nagasawa, H., Aikawa, M., Procell, P. M., Campbell, G. H., Collins, W. E., and Campbell, C. C. 1988. *Plasmodium malarie*: Distribution of circumsporozoite protein in midgut oocyst and salivary gland sporozoites. *Experimental Parasitology* **66,** 27–34.

Nardin, E. H., Nussenzweig, R. S., Nussenzweig, V., Harinasuta, K. T., Collins, W. E., Tapchaisri, P., and Chromcharn, Y. 1982. Circumsporozoite (CS) proteins of human malaria parasites *Plasmodium falcuparum* and *Plasmodium vivax. Journal of Experimental Medicine* **156,** 20–30.

Nussenzweig, V., and Nussenzweig, R. S. 1985. Circumsporozoite proteins of malaria parasites. *Cell* **42,** 401–403.

Ozaki, L. S., Svec, P., Nussenzweig, R. S., Nussenzweig, V., and Godson, G. N. 1983. Structure of the *Plasmodium knowlesi* gene coding for the circumsporozoite protein. *Cell* **34,** 815–822.

Posthuma, G., Meis, J. F. G. M., Verhave, J. P., Hollingdale, R. M., Ponnudurai, T., Meuwissen, H. E. T. J., and Geuze, H. J. 1988. Immunogold localization of circumsporozoite protein of the malaria parasite *Plasmodium falciparum* during sporogony in *Anopheles stephensi* midguts. *European Journal of Cell Biology* **46,** 18–24.

Ramsey, J. M., Salinas, E., Rodriguez, M. H., and Beaudin, R. L. 1994. Effects of transmission-blocking immunity on *Plasmodium vivax* infections in *Anopheles albimanus* populations. *Journal of Parasitology* **80,** 88–92.

Robson, K. J. H., Frevert, U., Reckmann, I., Cowan, G., Beier, J., Scargg, I. G., Takehara, K., Bishop, D. H. L., Pradel, G., Sinden, R., Saccheo, S., Muller, H. M., and Crisanti, A. 1995. Thrombospondin-related adhesive protein (TRAP) of *Plasmodium falciparum:* Expression during sporozoite ontogeny and binding to human hepatocytes. *EMBO Journal* **14,** 3883–3894.

Rosenberg, R., Wirtz, R. A., Lanar, D. E., Sattabongkot, J., Hall, T., Waters, A. P., and Prasittisuk, C. 1989. Circumsporozoite protein heterogeneity in the human malaria parasite *Plasmodium vivax. Science* **245,** 973–976.

Sam-Yellowe, T. Y. 1996. Rhoptry organelles of Apicomplexa: Their role in host cell invasion and intracellular survival. *Parasitology Today* **12,** 308–316.

Santoro, F., Cochrane, A. H., Nussenzweig, V., Nussenzweig, R. S., Gwadz, R. W., and Ferreira, A. 1983. Structural similarities among the protective antigens of sporozoites from different species of malaria parasites. *Journal of Biological Chemistry* **258,** 3341–3345.

Sina, B. J., Wright, C., Atkinson, C. T., Ballou, R., Aikawa, M., and Hollingdale, M. 1995. CSP-2: A protective antigen common to *Plasmodium falciparum* and *Plasmodium berghei. Molecular and Biochemical Parasitology* **69,** 239–246.

Sinden, R. E., and Garnham, P. C. C. 1973. A comparative study on the ultrastructure of *Plasmodium* sporozoites within the oocyst and salivary glands with particular reference to the incidence of the micropore. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **67,** 631–637.

Sinden, R. E., and Strong, K. 1978. An ultrastructurel study of the sporogonic development of *Plasmodium falciparum* in *Anopheles gambiae. Transactions of the Royal Society of Tropical Medicine and Hygiene* **72,** 477–491.

Sterling, C. R., Aikawa, M., and Vanderberg, J. P. 1973. The passage of *Plasmodium berghei* sporozoites through the salivary glands of *Anopheles stephensi:* An electron microscopy study. *Journal of Parasitology* **59,** 593–605.

Stewart, M. J., Schulman, S. and Vanderberg, J. P. 1985. Rhoptry secretion of membranous whorls by *Plasmodium berghei* sporozoites. *Journal of Protozoology* **32,** 280–283.

Stewart, M. J., and Vanderberg, J. P. 1991. Malaria sporozoites release circumsporozoite protein from their apical end and translocate it along their surface. *Journal of Protozoology* **38,** 411–421.

Stoute, J. A., Slaoui, M., Heppner, G., Momin, P., Kester, K. E., Desmons, P., Wellde, B. T., Garçon, N., Kryych, U., Marchand, M., Ballou, W. R., and Cohen, J. D. 1997. A preliminary evaluation of a recombinant circumsporozoite protein vaccine against *Plasmoidum falciparum* malaria. *The New England Journal of Medicine* **336,** 86–91.

Sultan, A. A., Thathy, V., Frevert, U., Robson, K. J. H., Crisanti, A., Nussenzweig, V., Nussenzweig, R., and Menard, R. 1997. TRAP is necessary for gliding motility and infectivity of *Plasmodium* sporozoites. *Cell* **90,** 511–522.

Tapchaisri, P., Chomcham, P., Poonthong, C., Asavanich, A., Limsuwam, S., Maleevan, O., Tharavanij, S., and Harinasuta, T. 1983. Anti-sporozoite antibodies induced by natural infection. *American Journal of Tropical Medicine and Hygiene* **32,** 1203–1208.

Touray, M. G., Warburg, A., Laughinghouse, A., Krettli, A. U., and Miller, L. H. 1992. Developmentally regulated infectivity of malaria sporozoites for mosquito salivary glands and the vertebrate host. *Journal of Experimental Medicine* **175,** 1607–1612.

Turner, D. P. 1981. Preliminary observations on the cell surface of *Plasmodium gallinaceum* sporozoites. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **757,** 176–178.

Vanderberg, J. P. 1975. Development of infectivity by *Plasmodium berghei* sporozoites. *Journal of Parasitology* **61,** 43–50.

Vermeulen, A. N., Van Munster, J. C., and Meuwissen, Th. J. H. E. 1982. *Plasmodium berghei:* Immunologically active proteins on the sporozoite surface. *Experimental Parasitology* **53,** 199–208.

Warburg, A., Touray, M., Krettli, A. V., and Miller, L. H. 1992. *Plasmodium gallinaceum:* Antibodies to circumsporozoite protein prevent sporozoites from invading the salivary glands of *Aedes aegypti. Experimental Parasitology* **75,** 303–307.

World Health Organization. 1967. "Chemotherapy of Malaria." Technical Report Series No. 375, p. 30. WHO, Geneva.

Wirtz, R. A., Burkot, T. R., Andre, R. G., Rosenberg, R., Collins, W. E., and Roberts, D. R. 1985. Identification of *Plasmodium vivax* sporozoites in mosquitoes using an enzyme immunosorbent assay. *American Journal of Tropical Medicine and Hygiene* **34,** 1048–1054.

Wirtz, R. A., Rosenberg, R., Sattabongkot, J., and Webster, H. K. 1990. Prevalence of antibody to heterologous circumsporozoite protein of *Plasmodium vivax. Lancet* **336,** 593–595.

Wirtz, R. A., Sattabongkot, J., Hall, T., Burkot, T. R., and Rosenberg, R. 1992. Development and evaluation of an enzyme linked immunosorbent assay for *Plasmodium vivax*-VK 247 sporozoites. *Journal of Medical Entomology* **29,** 845–857.

Yoshida, N., Potocnjak, P., Nussenzweig, V., and Nussenzweig, R. S. 1981. Biosynthesis of Pb 44, the protective antigen of sporozites of *Plasmodium berghei. Journal of Experimental Medicine* **154,** 1225–1236.

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