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Appearance and Localization of *Plasmodium yoelii* Circumsporozoite Protein during Sporogony in *Anopheles stephensi*

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ABSTRACT

The occurrence and distribution of circumsporozoite (CS) protein of *Plasmodium yoelii* 17X (lethal) was observed during sporogonic development in *Anopheles stephensi* mosquitoes. The CS protein was visualized by using a polyclonal antibody against synthetic peptides $(QGPGAP)_4$ of the immunodominant repeats of the CS protein and immunogold labeling. The CS protein is synthesized in immature oocysts from day 5 after infective blood meal when sporozoite formation has not yet started. The CS protein appears on the plasmalemma of oocysts and associates with the surface of sporoblasts and budding sporozoites. Micronemes and the pellicle of mature sporozoites were immunolabeled.

INTRODUCTION

Protective immunity to malaria can be obtained by immunization with irradiated sporozoites and it has been shown that the host antibody response to the sporozoite is mainly directed against the circumsporozoite (CS) protein (Nussenzweig and Nussenzweig 1984; Nardin et al. 1982; Zavala et al. 1983). The CS protein is believed to be involved in the infection of host hepatocytes (Hollingdale et al. 1987). Therefore, one of the primary strategies for malaria vaccine development has been to design subunit vaccines that induce protective levels of antibodies against the CS protein. The CS protein was found not only on the surface of salivary-gland sporozoites but also on the immature oocyst sporozoites (Potocnjak et al. 1980; Aikawa et al. 1981). These results were recently confirmed and expanded with monoclonal antibodies and immunogold technics (Aikawa et al. 1990; Hamilton et al. 1988; Nagasawa et al. 1987,1988; Posthuma et al, 1988). To know the onset and the site of CS protein synthesis in detail, we observed the appearance and localization of CS protein during sporogony by using an antibody against the synthetic peptide $(QGPGAP)_4$, one of the immunodominant repeat regions of the *P. yoelii* CS protein.

MATERIALS AND METHODS

P. yoelii oocysts and fixation: *Anopheles stephensi* mosquitoes were allowed to feed on ICR mice blood infected with *P. yoelii* 17X lethal(L) strain. The mosquitoes were collected 4–16 days after feeding and midguts were dissected into 1.0% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M PBS (pH 7.4) and fixed for 30 min at room temperature. Specimens were then washed three times in 0.1 M PBS (Aikawa & Atkinson 1990).

Antiserum: A synthetic peptide composed of four copies of Gln–Gly–Pro–Gly–Ala–Pro (QGPG AP)$_4$ and an additional Pro at the 5' end for structural stability was used as an antigen. The amino acid sequence of the synthetic peptide was derived from the DNA sequence of the immunodominant repeat of *P. yoelii* CS protein (Lal et al. 1987). Antisera were produced by immunizing rabbits with the synthetic peptide conjugated to bovine serum albumin (BSA).

Immunoelectron microscopy: Fixed mosquito midguts containing *P.yoelii* oocysts were dehydrated through a graded series of ethanol and embedded in LR White resin (London Resin Co Ltd). Sections were blocked for 30 min in 0.1 M PBS containing 5% nonfat dry milk, incubated overnight at 4°C in rabbit anti–synthetic peptide serum or pre–immune rabbit serum and then, incubated for 1 h with goat anti–rabbit IgG conjugated to gold particles (Janssen, Piscataway, NJ). Sections were stained with 2% uranyl acetate in 50% methanol and examined with a Hitachi H–800 electron microscope (Torii et al. 1989).

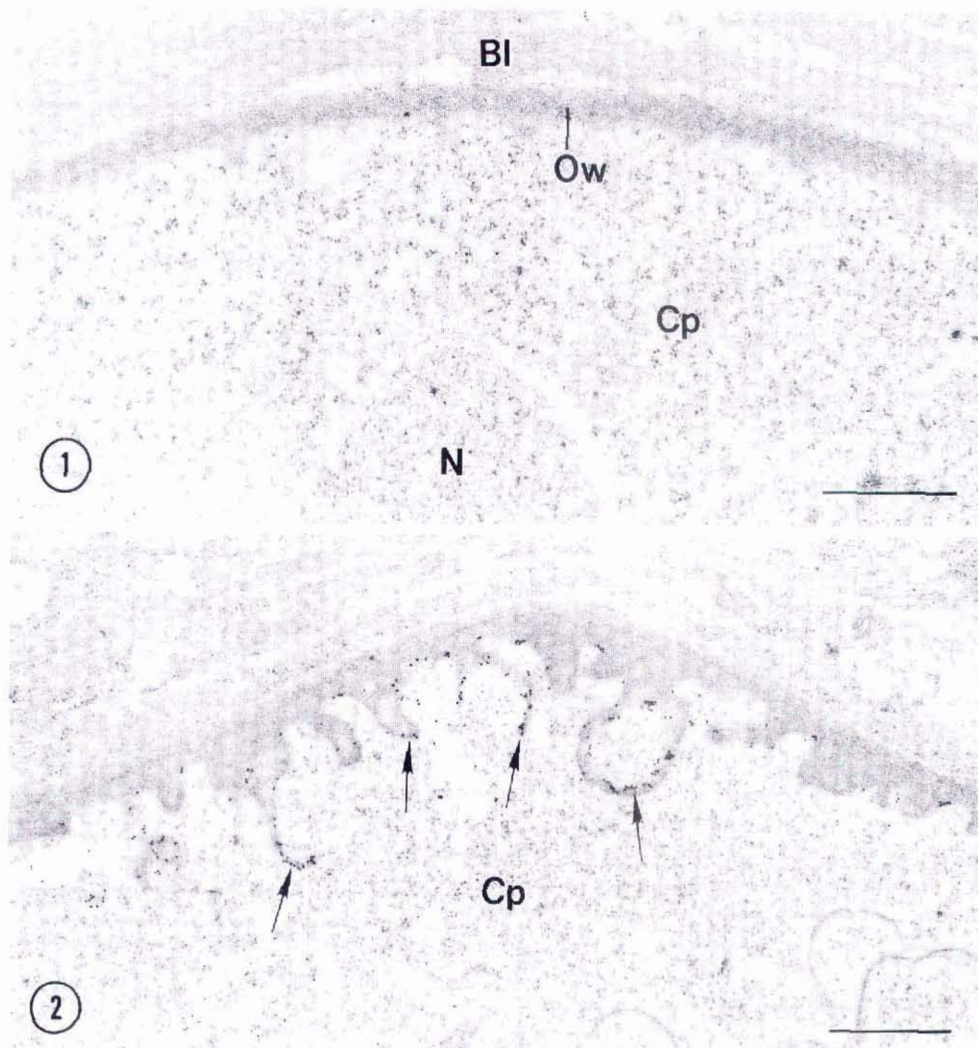

Fig. 1: Immature oocyst 4 days after infective feeding. Plasmalemma beneath the oocyst wall (Ow) is smooth. No immunolabeling is observed. Cp: Oocyst cytoplasm, Bl: basal lamina of midgut epithelium, N: nucleus. (Bar equals 1 μm)

Fig. 2: Five–day–old oocyst. Inner surface of oocyst wall (arrow), invaginating into the cytoplasm (Cp), is labeled with gold particles. (Bar equals 1 μm)

RESULTS

Mature oocysts containing fully developed sporozoites appeared between 8 to 12 days after the infective feeding, although oocyst development tends to occur asynchronously. Four-day-old oocysts were solid structures surrounded by a plasmalemma and an electron-dense wall. The plasmalemma was smooth and did not protrude into the oocyst cytoplasm. Little immunolabeling was observed on the plasmalemma or cytoplasm of the oocysts (Fig. 1). Five-day-old oocysts started to show synthesis of CS antigen. Gold label was seen in association with the plasmalemma which started to protrude into the cytoplasm. The amount of label present at this stage of development was rather small (Fig. 2). Six days after the blood meal, peripheral vacuoles were formed in the vicinity of the oocyst wall (Fig. 3A). The plasmalemma and the membrane of the peripheral vacuoles were heavily labeled with gold particles. Gold particles were also seen in association with the nuclear membranes and endoplasmic reticulum. A space was formed between the oocyst wall and the oocyst plasmalemma in 6- to 7-day-old oocysts (Fig. 3B). At the same time, cleft formation was observed

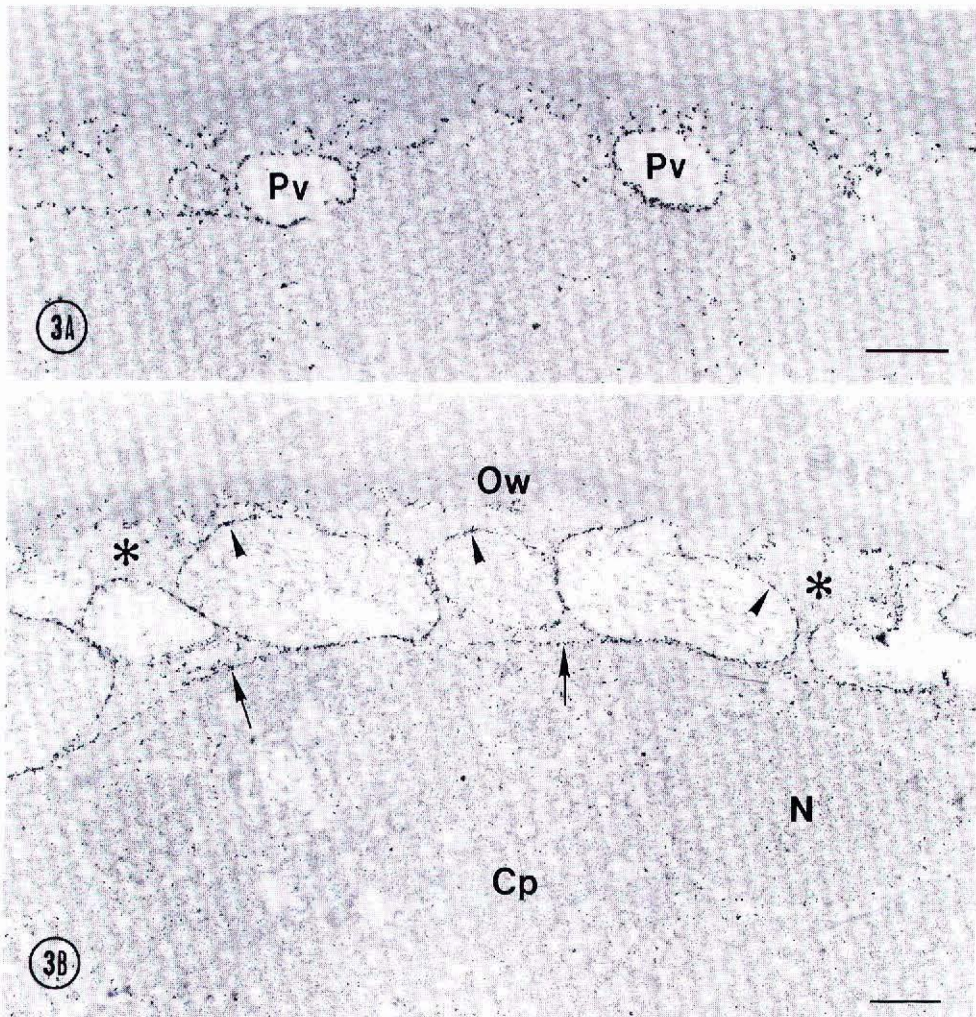

Fig. 3: Six-day-old oocyst. A: Peripheral vacuole (Pv) formation beneath the oocyst wall. B: The plasmalemma of oocyst (arrow) and the membrane of peripheral vacuoles (arrow head) are heavily labeled with gold particles. Subcapsular space (*) between the oocyst wall (Ow) and the solid cytoplasm (Cp) is present. N: nucleus. (Bar equals 1 μm)

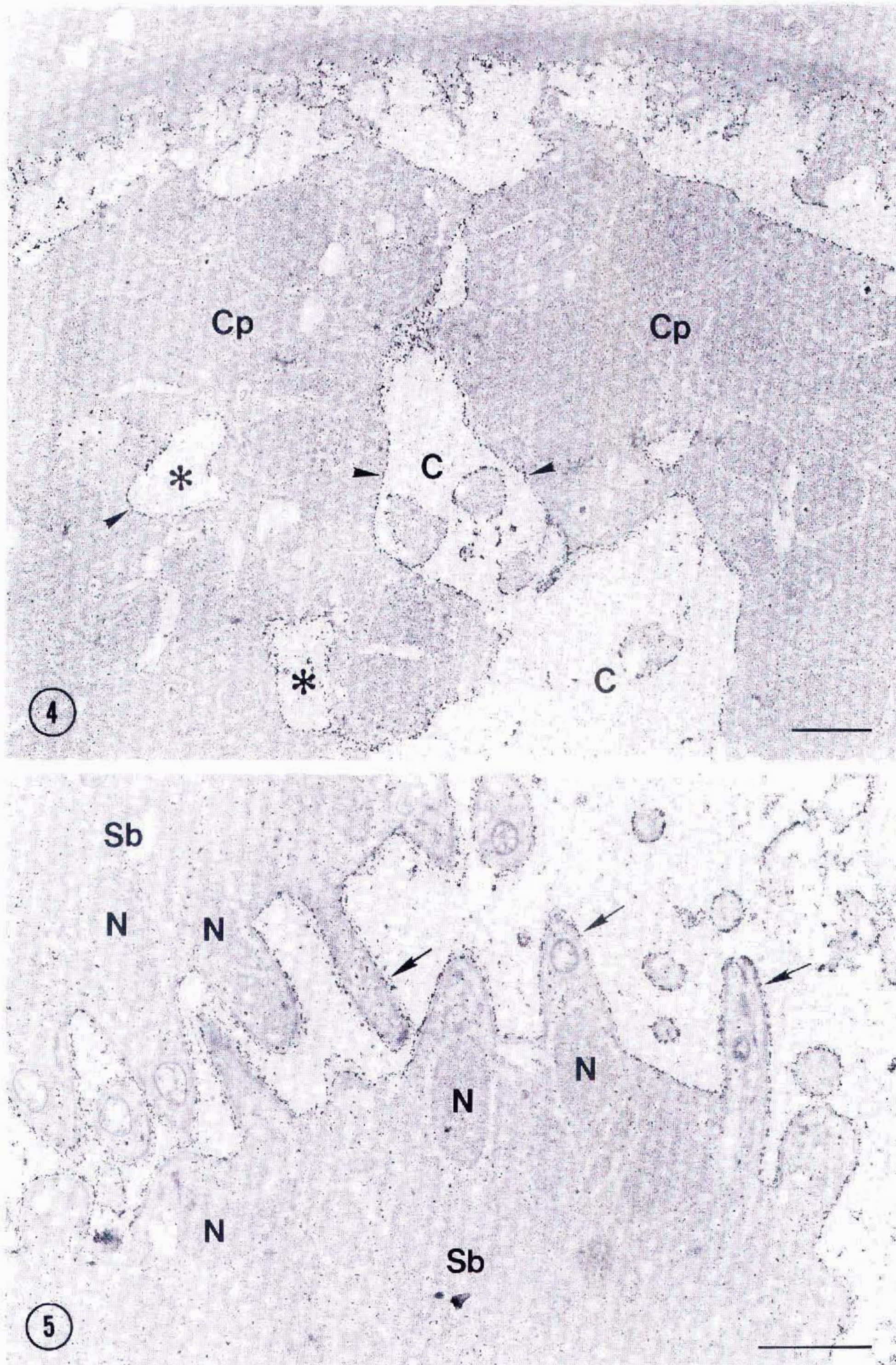

Fig. 4: Cleft formation in the seven–day–old oocyst. The cytoplasm of the oocyst (Cp) is subdivided by fused and expanded clefts (C). Cytoplasmic clefts (*) are also seen. Membranes of these clefts (arrow head) are immunolabeled. (Bar equals 2 μm)

Fig. 5: Budding sporozoites in the eight–day–old oocyst. Surface of the budding sporozoites (arrow) is immunolabeled. Sb: sporoblast, N: nucleus. (Bar equals 2 μm)

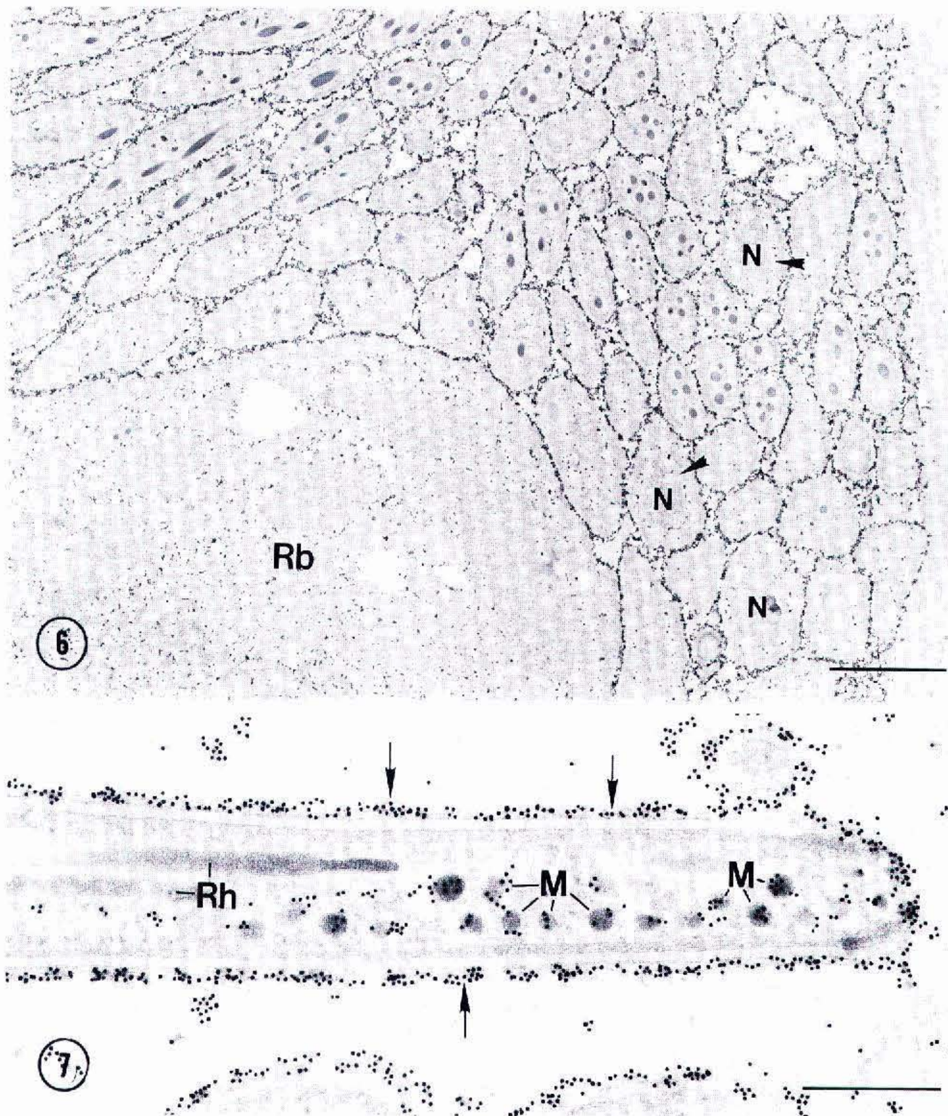

Fig. 6: Ten-day-old oocyst which is filled with sporozoites and a residual body (Rb). Surface of the sporozoites and residual body (Rb) is labeled with gold particles. Nuclear envelope (arrow head) of the sporozoites is also immunolabeled. N: nucleus. (Bar equals 2 μm)
Fig. 7: Mature sporozoites in the twelve-day-old oocyst. The pellicle (arrow) and the micronemes (M) are labeled with gold particles. Rhoptries (Rh) are not labeled. (Bar equals 0.5 μm)

in the solid cytoplasm. The membrane of these clefts showed immunoreactivity indicating CS protein synthesis. As cleft formation proceeded, the size of the clefts increased. The membrane of the clefts finally fused with the plasmalemma and invaginations of the oocyst cytoplasm were formed (Fig. 4). The cytoplasm was subdivided into sporoblasts by these invaginations. The surface membrane of the sporoblast was heavily labeled with gold particles. At day 8, sporozoites started to bud on the sporoblast surface and the CS protein was transferred to the surface of the budding sporozoites (Fig. 5). When sporogony was completed about 10 days after the blood meal, the oocyst was filled with mature sporozoites and one or more residual bodies (Fig. 6). The pellicle of the mature sporozoites was densely labeled, and the nuclear envelope was labeled with gold particles. The micronemes in the sporozoites also showed immunoreactivity, but rhoptries were not immunolabeled (Fig. 7).

DISCUSSION

During the past decade, the primary strategy for malaria sporozoite vaccine development has been to produce subunit vaccines that induce antibodies to the repeat regions of the CS protein (Young et al., 1985; Ballou et al., 1985; Mazier et al., 1986) and, more recently, stimulate CS protein-specific T-cell activity which prevent effective sporozoite invasion of hepatocytes (Romero et al., 1989; Weiss et al., 1990). Synthesis and distribution of CS protein during sporogony has been described for several species of Plasmodia by using monoclonal antibodies and immunoelectron microscopy (Aikawa et al. 1990; Hamilton et al. 1988; Nagasawa et al. 1987, 1988; Posthuma et al. 1988). According to these reports, CS protein begins to appear early in sporogony before segmentation of the oocyst. Posthuma et al. (1988) and Aikawa et al. (1990) observed the synthesis of CS antigen in 6- and 7-day-old oocysts, respectively. This finding is supported by indirect fluorescent antibody tests showing that oocysts start to react with anti-CS protein monoclonal antibodies from 7 days after the infective blood meal (Verhave et al. 1988). The present study has demonstrated the appearance of CS immunoreactivity as early as 5 days after the infective blood meal. At this stage, CS immunoreactivity is present on the oocyst plasmalemma. CS immuno-reactivity was also observed on the membrane of the peripheral vacuoles. As suggested by Nagasawa et al. (1988), this immunoreactivity may indicate the appearance of precursors to the CS protein to their incorporation into the sporoblast membrane.

Posthuma et al. (1988) suggested that sporoblasts were formed by the invagination of the plasmalemma and penetration into the solid cytoplasm of the oocysts. We demonstrated the appearance of cytoplasmic clefts in the solid oocyst cytoplasm by immunogold labeling. The clefts fused to each other, increased in size, and finally fused with the plasmalemma of the oocyst. We reconfirm previous studies by transmission electron microscopy (Sinden and Strong 1978) with these findings, and believe that invaginations between the sporoblasts are formed by fusion and expansion of the cytoplasmic clefts.

We observed abundant CS immunoreactivity in all three pellicular membranes of mature oocyst sporozoites. Similar immunoreactivity has been described on mature oocyst sporozoites of *P. malariae*, *P. ovale*, and *P. berghei* (Hamilton et al. 1988; Nagasawa et al. 1987, 1988). In mature oocysts of *P. falciparum*, however, only the external pellicular membrane of the sporozoite is labeled. By contrast, all three pellicular membranes of *P. falciparum* sporozoite display abundant CS immunoreactivity in the salivary gland (Posthuma et al. 1988, 1989). These findings might indicate that sporozoite maturation is different for *P. falciparum*.

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