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Localization of CS and non-CS antigens in the sporogonic stages of *Plasmodium yoelii**

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Monoclonal antibodies (MAbs) and colloidal gold probes were used to localize circumsporozoite (CS) protein and two unrelated polypeptides in developing oocysts and salivary gland sporozoites of the 17X (NL) strain of Plasmodium yoelii. *MAbs NYS1, NYS2, and NYS3 recognized different epitopes of the* P. yoelii *CS protein and produced similar patterns of immunolabelling on developing oocysts and sporozoites. A small percentage of oocysts contained developing sporoblasts and sporozoites that did not exhibit surface reactivity to MAbs NYS1, NYS2 or NYS3, although internal labelling was associated with endoplasmic reticulum (ER). These sporozoites were still capable of completing development and invading salivary glands where they could be found adjacent to sporozoites with densely labelled surface coats. If these sporozoites are infective, their presence may explain in part the failure of CS vaccines to completely protect immunized animals against challenge. The non-CS antigen recognized by MAbs NYS4 did not become abundant until late in sporogony. Some gold labelling was associated with the surface of budding and mature sporozoites, but the antigen was most abundant within the cytoplasm and micronemes. A second non-CS antigen identified by NYS5 first appeared in 7-day-old oocysts, although labelling was sparse. Small quantities of antigen appeared on the sporoblast membrane, cytoplasmic clefts and ER of oocysts and was associated with micronemes and the surface of budding and mature sporozoites. As the role played by non-CS antigens in the biology of the parasite is not yet known, further characterization of their function is needed before their potential as vaccine candidates can be determined.*

Introduction

Charoenvit and associates (1) produced monoclonal antibodies (MAbs) against sporozoites of *Plasmodium yoelii*. MAbs NYS1, NYS2 and NYS3 recognized the CS antigen, although each reacts with a different epitope. The remaining 2 MAbs, NYS4 and NYS5, recognized antigens which are not CS protein. The antigen recognized by NYS4 is a 140 kDa protein (relative molecular mass, M_r, 140 000) with a common determinant with the CS protein. The antigen recognized by NYS5 could not be found by Western blotting. Immunofluorescent microscopy (IFA) demonstrated that 3 MAbs (NYS1, NYS2 and NYS3) reacted with the sporozoite surface, although there were slight differences in patterns of reactivity. On the other hand, NYS4 produced a large patchy pattern that appeared to be both internal and on the surface of the sporozoite. MAb NYS5 produced a pattern that appeared to be on the surface but was restricted to polar regions. The precise locations of antigens detected by these MAbs were difficult to determine because of the limited resolution of IFA. Therefore, we performed immunoelectron microscopy with particular attention to non-CS proteins, since we do not yet know what role these antigens play in the biology of the parasite.

Materials and methods

Since detailed descriptions of the production of MAbs, ascitic fluids, IFA test, CSP reaction, SDS-PAGE and immunodetection and sporozoite neutralization have already been published (1), these methods will not be described here.

Immunoelectron microscopy was performed on 4-, 7-, 10- and 15-day-old oocysts and salivary gland sporozoites of the 17X (NL) strain of *P. yoelii*. Infected midguts and salivary glands were fixed for 20 minutes at 4 °C in 1% paraformaldehyde, 0.1% glutaraldehyde in 100 mmol/l phosphate buffer, pH 7.4 and were embedded at low temperatures in LR gold resin

* The opinions and assertions herein are those of the authors and are not to be construed as official or as reflecting the views of the US Navy or the Naval service at large. The experiments reported herein were conducted according to the principles set forth in the "Guide for the care and use of laboratory animals" Institute of Laboratory Animal Resources, National Research Council (DHHS Publ. (NIH) 86-23), 1985.

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(London Resin Company). Sections were cut with a diamond knife, mounted on nickel grids and labelled with MAbs NYS1, NYS2, NYS3, NYS4 and NYS5 and 15 nm immunoglobulin-gold as described elsewhere (*2*).

Results

Localization of CS protein by NYS1, NYS2 and NYS3

NYS1, NYS2 and NYS3 produced similar results when tested on 4-, 7- and 10-day-old oocysts and salivary gland sporozoites of *P. yoelii*.

Four-day-old oocysts did not show subcapsular vacuolization, cleft formation, or budding sporozoites. Little label was associated with the cytoplasm. Seven-day-old oocysts started to show synthesis of CS antigen. Gold label was seen in association with the nuclear membranes, endoplasmic reticulum and plasma membrane (Fig. 1). The subcapsular space of developing oocysts also was labelled with gold particles. In some oocysts, the sporoblast cytoplasm had started to contract, showing deep clefts in its peripheral cytoplasm. Gold particles were seen on the surface of clefts and on fine granular electron-dense material in the subcapsular space. There were many sporozoites in 10-day-old oocysts. Gold particles were present mainly on the surface of sporozoites but some particles were also seen within the sporozoite cytoplasm (Fig. 2). The residual sporoblast cytoplasm was also labelled with gold particles. Membrane-bound electron-opaque granules were also labelled with gold particles.

Mature sporozoites in salivary glands of infected mosquitos were coated evenly with CS protein (Fig. 3). Label was also associated with micronemes (Fig. 3). Electron-opaque secretory material which surrounded the sporozoite was also labelled with gold particles.

A small percentage (< 5%) of oocyst and salivary gland sporozoites did not exhibit surface reactivity with any of the 3 MAbs to the CS protein (Fig. 4 and 5). A few gold particles were found on internal micronemes, on perinuclear membranes of budding sporozoites, and on endoplasmic reticulum in the sporoblast cytoplasm. Oocysts that contained these "abnormal" sporozoites were few in number, but uniform in their formation of sporozoites that lacked surface labelling, i.e., they did not contain mixed populations of labelled and unlabelled sporozoites. These oocysts were observed in the same section next to oocysts that produced sporozoites with densely labelled surface coats.

Fig. 1. **Section of a 7-day-old *Plasmodium yoelii* oocyst incubated with MAb NYS1 against CS protein and immunoglobulin-gold.** Gold particles are associated with the endoplasmic reticulum (ER), cytoplasmic matrix and subcapsular areas (arrow) × 20 000

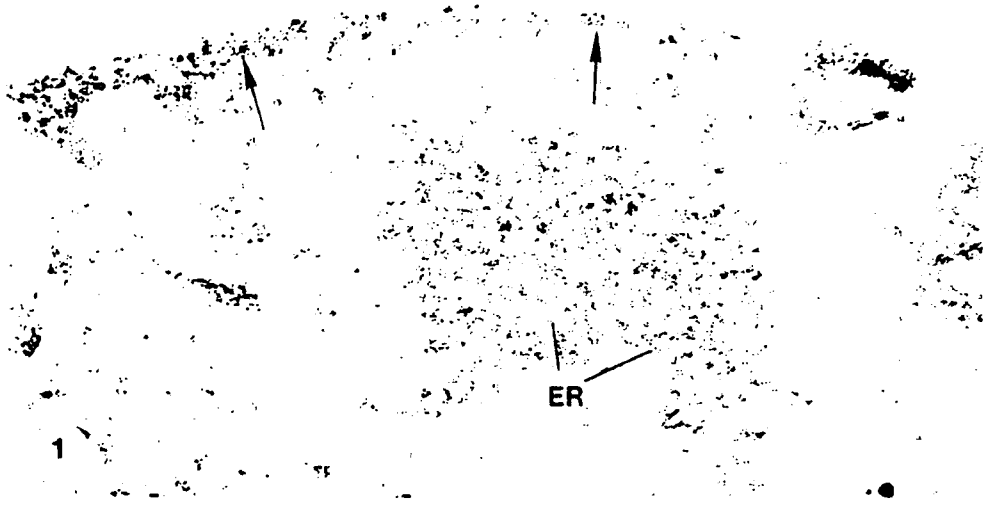
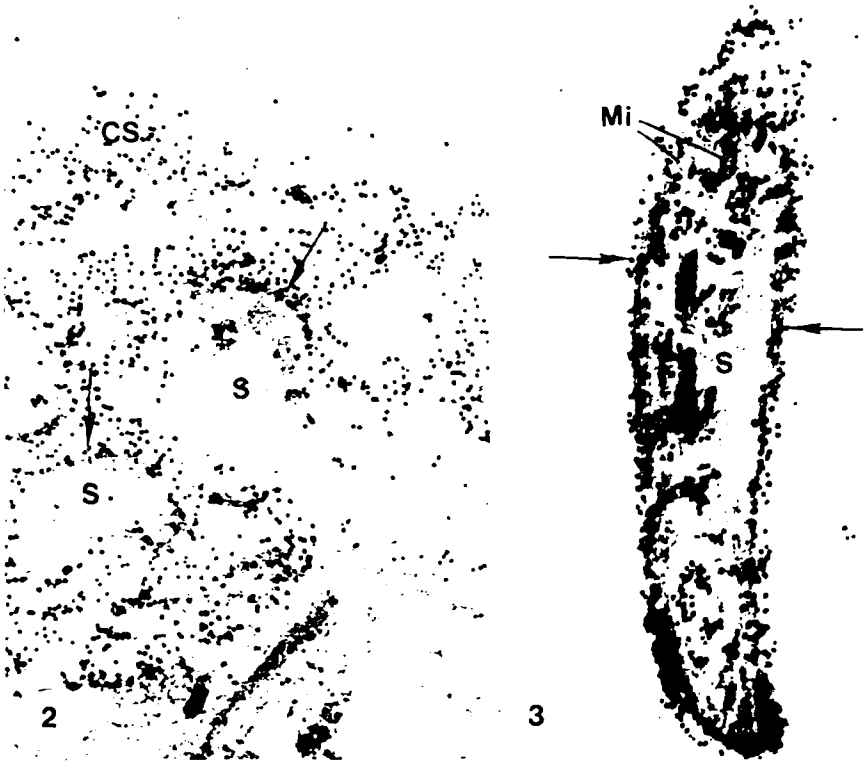


Fig. 2. **Section of a 10-day-old oocyst incubated with MAb NYS2 against CS protein and immunoglobulin-gold.** Gold label (arrow) is associated with the surface of clefts and of budding sporozoites (S). The oocyst capsule space (CS) is also labelled with gold particles. × 35 000

Fig. 3. **Section of a salivary gland sporozoite (S) incubated with MAb NYS3 against CS protein and immunoglobulin-gold.** Gold label is associated with the surface (arrow), and micronemes (Mi). × 44 000



Localization of non-CS protein by NYS4 (NYS4-non-CS protein)

Only a few random gold particles were associated with 6- and 7-day-old oocysts. No labelling was found in the endoplasmic reticulum indicating that synthesis of this antigen had not started. Synthesis of NYS4-non-CS protein began late in sporogony, when budding of sporozoites started (Fig. 6). Dense labelling of gold particles was associated with the endoplasmic reticulum and with small membrane-bound, electron-opaque granules in the sporoblast cytoplasm of 15-day-old oocysts (Fig. 6). These small membrane-bound electron-opaque granules appeared to migrate into budding sporozoites. When salivary gland sporozoites were examined, micronemes, but not rhoptries, were densely labelled by MAb NYS4 (Fig. 7). Some scattered labelling was seen on the plasma membrane of sporozoites.

Localization of non-CS protein by NYS5 (NYS5-non-CS protein)

Similar to NYS4-non-CS protein, only a few gold particles were present in the sporoblast cytoplasm of the 7-day-old oocysts. Antigen recognized by NYS5 appeared on the surface and cytoplasm of sporoblasts in 10-day-old oocysts (Fig. 8). Gold particles were seen in the cytoplasmic matrix as well as in the endoplasmic reticulum. Gold particles were associated with the micronemes and cytoplasm of salivary gland sporozoites as well as on the surface (Fig. 9).

Fig. 4. **Section of a 10-day-old oocyst incubated with MAb NYS1 against CS protein and immunoglobulin-gold.** Gold particles are only associated with perinuclear membranes (arrow) and endoplasmic reticulum (ER). Label is not on the surface (S) of the sporoblast or budding sporozoites. × 21 000

Fig. 5. **Section of two salivary gland sporozoites incubated with MAb NYS2 against CS protein and immunoglobulin-gold.** Sporozoite at the right (S2) is labelled on the surface (arrow) and micronemes (Mi). The sporozoite at the left (S1) shows gold label particles only over micronemes (Mi). × 40 000

Discussion

The distribution of circumsporozoite protein in oocysts and salivary gland sporozoites has been studied by immunoelectron microscopy in *P. malariae*, *P. ovale*, *P. knowlesi*, *P. berghei* and *P. falciparum* [3–7]. Based on these data, synthesis of CS protein appears early in sporogony before the differentiation of sporozoites. CS protein becomes abundant in the oocyst cytoplasm before sporozoites begin to bud. The presence of CS antigen in the subcapsular space

Fig. 6. **Section of a 15-day old oocyst incubated with MAb NYS4 against non-CS protein and immunoglobulin-gold.** Dense labelling of gold particles is mainly associated with membrane-bound electron-dense granules (D). These granules are also in the cytoplasm of budding sporozoites (S). A few scattered gold particles (arrows) are present on the surface of budding sporozoites. × 40 000

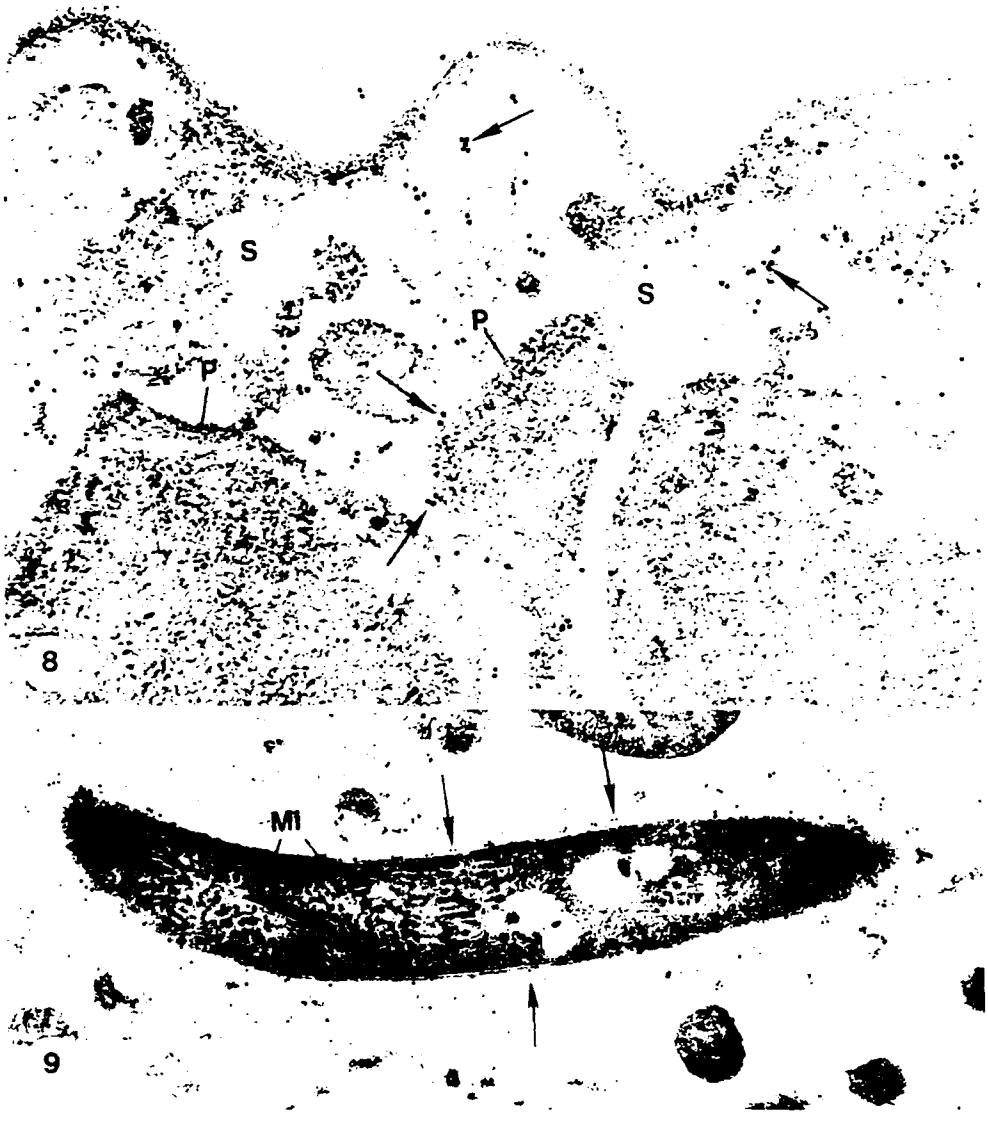
Fig. 7. **Section of a salivary gland sporozoite incubated with MAb NYS4 against non-CS protein and immunoglobulin-gold.** Abundant gold particles are associated with sporozoite cytoplasm and micronemes (Mi). Some scattered labelling (arrows) is associated with the sporozoite surface. × 45 000

might indicate sloughing of CS antigen from the surface of the oocysts.

The presence of CS antigen in the micronemes has been described in *P. knowlesi*, *P. malariae*, and *P. berghei* (*3*, *5*, *6*). In addition, rhoptries of *P. knowlesi* were also shown to possess CS protein (*5*). However, cross-reactivity of MAb NYS2 with NYS4-non-CS protein on Western blots might indicate that labelling of micronemes in *P. yoelii* sporozoites could be a cross-reaction (*1*).

Fig. 8. **Section of a 10-day-old oocyst incubated with MAb NYS5 against non-CS protein and immunoglobulin-gold.** Only a few gold particles (arrows) are associated with the cytoplasmic matrix, plasma membrane (P), and subcapsular spaces (S). × 37 000

Fig. 9. **Section of a salivary gland sporozoite incubated with MAb NYS5 against non-CS protein and immunoglobulin-gold.** Gold particles are associated with micronemes (Mi), cytoplasm, and the sporozoite surface (arrow). × 30 000



It is interesting that a small percentage of oocyst and salivary gland sporozoites were not labelled on their surface by any of 3 MAbs to the CS protein. We do not know if these sporozoites are capable of being transmitted or whether they are infective. If they are infective, their presence may explain, in part, why immunization with sporozoite vaccines is not completely protective (*8–10*). It is unclear what molecules are on the surface of these sporozoites. Presence of internal reactivity with MAbs to the CS protein suggests that the antigen is not exported to the surface of the developing sporozoites. Alternatively, only a small fragment of the CS antigen may be exported to the surface or a new antigenic variant that is not recognized by any of the 3 MAbs may have arisen. Significantly, Rosenberg and others (*11*) found that over 14% of clinical cases of *P. vivax* in western Thailand produced immunologically distinct sporozoites. These sporozoites had CS protein with a different repeating nonapeptide that did not bind antibodies either to *P. vivax* sporozoites from different geographic areas or to a recombinant CS protein that is a potential vaccine candidate. Since the CS protein is believed to play such an important role in sporozoite motility and host cell recognition and invasion, it is clear that this phenomenon needs to be investigated further.

NYS4-non-CS protein appeared in the oocyst much later than CS protein detected by NYS1, NYS2 and NYS3. The NYS4-non-CS protein was first evident in oocysts with budding sporozoites and was always associated with small membrane-bound electron-opaque granules in the oocyst cytoplasm. These granules appear to migrate to the budding sporozoites, carrying NYS4-non-CS protein into the sporozoites. These granules might be precursors of micronemes, since their size and shape are similar to micronemes and as micronemes of the sporozoite also contain NYS4-non-CS protein. Some scattered labelling was also associated with the surface of sporozoites. This may correspond to the patchy labelling observed by immunofluorescent microscopy.

The distribution of NYS5-non-CS protein is similar to that of NYS4-non-CS protein. Both antigens are mainly located in micronemes and sporozoite cytoplasm although NYS5-non-CS protein is on the surface of the sporozoite. Also, they appear late in the oocyst. However, NYS5-non-CS protein was not associated with small membrane-bound electron-opaque granules in the oocyst. This may indicate that synthesis of NYS5-non-CS protein is different from that of NYS4-non-CS protein.

Our immunoelectron microscopy demonstrated that the distribution of NYS4-non-CS protein of *P. yoelii* is different from that of CS protein. Since the role of non-CS proteins in the biology of the parasite is still not clear, further investigation of their function may be required before their potential as vaccine candidates can be determined.

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