Presence of a circumsporozoite-like protein in micronemes of blood-stage merozoites of malaria parasites

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We demonstrate for the first time the presence of a circumsporozoite (CS)-like protein in invasive blood stages of malaria parasites. Immunogold electron microscopy using antisporozoite monoclonal antibodies localized these antigens in the micronemes of merozoites. Western immunoblot and two-dimensional gel electrophoresis of mature blood-stage extracts of Plasmodium falciparum, P. berghei, P. cynomolgi, *and* P. brasilianum *identified polypeptides having the same apparent molecular mass and isoelectric points as the corresponding sporozoite (CS) proteins. The CS-like protein of merozoites is present in relatively minor amounts, compared to the CS protein of sporozoites. Mice with long-term* P. berghei *blood-induced infections develop antibodies which react with sporozoites.*

Introduction

Sporozoites are the invasive stage of plasmodia inoculated into the vertebrate host by the bite of *Anopheles* mosquitos. Their surface membrane is covered by the circumsporozoite (CS) protein which arises from higher M_r (relative molecular mass) precursors. All CS proteins contain an immunodominant region consisting of a single epitope which is tandemly repeated and species specific. Monoclonal antibodies (mAbs) directed against this repeat region inhibit sporozoite invasion of target cells *in vitro* and their passive administration protects mice against sporozoite challenge (reviewed in *1*). Synthetic peptides and recombinant CS proteins based on the immunodominant epitope of *Plasmodium falciparum* have been used in human immunization trials and have induced some degree of protection (*2*, *3*).

Merozoites are the invasive stage of plasmodia released by rupture of infected red blood cells (IRBC) containing mature parasites. Ultrastructural data suggest that merozoite entry into the RBC is mediated by contents of rhoptries which are tear-shaped organelles located anteriorly in both merozoites and sporozoites (*4*). Microneme and rhoptry contents appear to be antigenically distinct (reviewed in *5*). Microneme antigens are poorly characterized but have been shown to be released into the medium during *in vitro* growth of IRBC (*6*). Recently, the CS protein and/or its precursors have been demonstrated in the micronemes of sporozoites (*7*).

Methods and results

CS proteins, like the protective response they induce, have been considered to be stage specific (reviewed in *1*). In the present study, using antisporozoite mAbs we have demonstrated, for the first time, the presence of CS-like proteins in the micronemes of blood-stage merozoites of *P. falciparum* (NF 54 strain), *P. berghei* (NK 65 strain), *P. cynomolgi* (Berok strain), and *P. brasilianum* (Colombian strain). This finding cannot be explained by "carry over" of the antigen from sporozoites or exoerythrocytic stages since all parasites that were used were derived after several passages of blood-induced infections.

Immunogold electron microscopy (*8*) was performed using several *P. brasilianum* antisporozoite mAbs which react with the immunodominant repeat sequence of the CS protein of *P. brasilianum* and *P. malariae* sporozoites. Gold particles were clustered over micronemes of budding merozoites within the segmenters but not over their rhoptries. There was no labelling of young parasites or of the cytoplasm or membrane of the IRBC. Using the same mAbs, we also detected microneme as well as surface membrane localization of the CS antigen in sporozoites.

Extracts of sporozoites and RBC containing schizonts/segmenters of *P. falciparum*, *P. berghei*, *P. cynomolgi*, and *P. brasilianum* were analysed by Western immunoblot (*8*). For each species, the major merozoite antigen detected by the homologous antisporozoite mAb had the same M_r as did the corresponding CS protein precursor. A weaker band having

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the same M_r as the mature CS protein was also detected in blood-stage extracts of *P. berghei* and *P. brasilianum*.

The isoelectric points (pI) of the merozoite CS-like antigens were compared with the PI of the corresponding CS proteins of sporozoites using two-dimensional gel electrophoresis (*9*) and Western immunoblot. For *P. brasilianum*, a single antigen was detected, the pI of which corresponded exactly to the pI of the CS precursor molecule. For *P. berghei*, the PIs of the two merozoite-associated CS-like antigens were exactly the same as those of the CS protein and its precursor.

Our preliminary data, obtained using extract of schizonts/segmenters of *P. cynomolgi* metabolically labelled with ^{35}S-methionine, indicate that the CS-like protein is actively synthesized during development of blood stages. Two proteins, having the same electrophoretic mobilities as the *P. cynomolgi* CS protein and its precursor, were precipitated from the radiolabelled blood-stage extract by the homologous antisporozoite mAb.

Characteristic of the immunodominant epitope of the CS protein of sporozoites, the CS-like molecules found in merozoites of *P. falciparum*, *P. cynomolgi*, *P. brasilianum*, and *P. berghei* contain an epitope which is represented more than once as determined by a two-site one-antibody immunoradiometric assay (*10*).

The precise amount of CS-like protein in merozoites is difficult to quantitate because it appears only in some, and not all, mature schizonts. Furthermore, the amount of CS-like protein appears to vary in different plasmodial species, with *P. brasilianum* having the greatest amount. For this species we have estimated, using an IRMA and extracts of mostly mature parasites, that the amount of CS-like protein in each segmenter is approximately 100- to 1000-fold less than the amount of CS protein in each sporozoite (AHC, unpublished data). Studies are now in progress to assess the presence of mRNA transcripts for the CS protein in blood stages and to characterize the mechanisms which regulate the stage-related level of expression of this protein.

To determine if the CS-like protein is immunogenic and can induce the formation of antibodies which react with sporozoites, we blood-induced *P. berghei* infections in mice and allowed the mice to develop a long-term infection of high parasitaemia. Immune sera of these mice were reactive in the CSP assay, by immunofluorescence using glutaraldehyde-fixed sporozoites as antigen, and by an IRMA using as antigen the *P. berghei* recombinant CS protein (*11*). In addition, by Western blot, immune sera of several of these mice detected the CS protein (Pb44) and its precursor.

The fact that mice with long-term blood-induced *P. berghei* infections produced some antibodies which react with the corresponding sporozoites raises the possibility that the merozoite CS-like antigen contributes to the antisporozoite antibody response of individuals living in malaria endemic areas. This does not appear to be a major contributing factor since serological studies in endemic areas have shown low antisporozoite antibody responses in young children with high levels of parasitaemia (*12*), and no significant differences in the levels of antisporozoite antibodies in the presence or absence of chemoprophylaxis (*13*).

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