

CIRCUMSPOROZOITE PROTEINS OF MALARIA PARASITES CONTAIN A SINGLE IMMUNODOMINANT REGION WITH TWO OR MORE IDENTICAL EPITOPES*

BY FIDEL ZAVALA, ALAN H. COCHRANE, ELIZABETH H. NARDIN,
RUTH S. NUSSENZWEIG, AND VICTOR NUSSENZWEIG

*From the Departments of Microbiology (Division of Parasitology) and Pathology, New York University
Medical Center, New York 10016*

Protective immunity against several species of malaria parasites has been obtained by vaccination with x-irradiated sporozoites (reviewed in reference 1). The serum of protected animals reacts with the surface membrane of sporozoites and neutralizes their infectivity, which suggests that the effect of the vaccine is at least in part antibody mediated. The membrane-associated protective antigens of sporozoites of rodent (2, 3), simian (4), and human (5) malaria have recently been identified by means of monoclonal antibodies (mAb).¹ They are stage-specific proteins that are distributed uniformly over the entire surface of the parasite and are shed when cross-linked by antibodies (3).

Evidence that these circumsporozoite (CS) proteins of different malaria species are structurally, antigenically, and functionally related molecules has been presented elsewhere (6). Of particular interest were the observations that Fab fragments of a mAb against the CS protein of *Plasmodium berghei* inhibited the attachment and interiorization of these sporozoites into target cells (7). These findings raised the intriguing possibility that some of the epitopes recognized by the mAb might either coincide with or be in close proximity to the areas of the CS molecules that are involved in parasite penetration.

To investigate this phenomenon further we compare here the specificities of a series of mAb against CS proteins of parasites of monkey (*P. knowlesi*) and human (*P. vivax* and *P. falciparum*) malaria, including some that neutralized the infectivity of the sporozoites in vivo (4, 5) or in vitro.² We present evidence that in every instance the mAb recognize the same unique region of the CS molecule, which contains at least two identical epitopes.

Materials and Methods

Monoclonal Antibodies. The production and characterization of mAb against sporozoites of *P. knowlesi*, *P. falciparum*, and *P. vivax* are described elsewhere (4, 5). The mAb were partially

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¹ Abbreviations used in this paper: BSA, bovine serum albumin; CS, circumsporozoite; mAb, monoclonal antibody; NP-40, Nonidet P-40; OVA, ovalbumin; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; STI, soybean trypsin inhibitor.

² Hollingdale, M. R., E. H. Nardin, S. Tharavani, A. L. Schwartz, and R. S. Nussenzweig. Inhibition of entry of *Plasmodium falciparum* and *P. vivax* sporozoites into cultured cells as an in vitro assay of protective antibodies. Manuscript submitted for publication.

purified from ascites fluids by ammonium sulfate precipitation followed by chromatography on Sephadex G200.

Sporozoites. Sporozoites of *P. vivax* and *P. falciparum* (isolates from Thailand), as well as *P. knowlesi* (H strain), were obtained from mosquito salivary glands 14–17 d after an infective blood meal (8). Sporozoite extracts were prepared by placing a known number of sporozoites in 500 μ l of phosphate-buffered saline containing 1% bovine serum albumin (PBS-BSA). Protein inhibitors were added as previously described (2). Nonidet P-40 (NP-40) was then added to a final concentration of 0.5%, and the mixtures were incubated at room temperature for 1 h. After centrifugation at 100,000 *g* for 1 h, the supernatants were stored at -70°C .

Antigen-coated Microtiter Plates. Sporozoites ($6 \times 10^4/\text{ml}$) were suspended in PBS containing protease inhibitors. 50 μ l of this suspension was placed in wells of polyvinylchloride flexible microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA). After freezing (-70°C) and thawing the plates six times, they were stored at -70°C . Before using, the wells were washed three times with PBS-BSA and kept in this solution for 1 h at room temperature.

Competitive Binding Assays Between Monoclonal Antibodies. Wells of antigen-coated plates were incubated with 30 μ l of cold mAb at a concentration of 50 $\mu\text{g}/\text{ml}$ in PBS-BSA. After 1 h of incubation, 10 μ l of one of the ^{125}I -labeled mAb (1–10 ng, 10^4 – 10^6 cpm) was added to the wells, and the incubation proceeded for an additional hour. Preliminary experiments showed that the above concentration of cold mAb was 25–50 times greater than that required to inhibit completely the binding of the hot homologous mAb to the antigen-coated plates. The wells in the plates were then washed three times with PBS-BSA, dried, and counted in a gamma counter. This procedure was repeated for every possible combination of hot vs. cold mAb against a given CS protein. As negative controls, antigen-coated wells were incubated either with an unrelated mAb or with PBS-BSA before the addition of the labeled mAb. The number of specific counts bound to antigen in the absence of inhibitor mAb was calculated as the difference between the number of counts bound to the wells incubated with the labeled mAb in the presence of PBS-BSA and the number of counts that could not be inhibited by an excess of the homologous, cold mAb, the latter representing nonspecific binding. From these values the percent inhibition of specific binding was calculated for each cold mAb. Results represent the means of duplicates.

Competitive Binding Assays Between mAb and Anti-Sporozoite Immune Sera. Immune serum anti-*P. knowlesi* sporozoites from rhesus monkeys was obtained by repeated immunizations with irradiated sporozoites administered intravenously (9). Human immune sera anti-*P. vivax* and anti-*P. falciparum* were obtained from volunteers exposed to multiple bites of irradiated, infected mosquitoes and shown to be protected against sporozoite challenge (10, 11). The anti-*P. knowlesi* immune sera were absorbed with salivary glands of uninfected mosquitoes. 100 μ l of immune serum diluted 1:10 in PBS-BSA was incubated with 100 packed salivary glands. After 2 h incubation at room temperature, the sample was centrifuged (8,000 *g*, 5 min) and the supernatant was used in the assay.

Anti-sporozoite antibodies were detected by incubating dilutions of the immune sera in wells of microtiter plates coated with homologous antigen. After 2 h incubation at room temperature, the wells were washed with PBS-BSA. Then an excess of affinity-purified, ^{125}I -labeled IgG of rabbit anti-monkey or rabbit anti-human immunoglobulin (Ig) was added to detect the presence of Ig bound to the immobilized antigen.

The competitive binding assay between mAb and the immune sera was performed as above, except that before the addition of the immune sera, the antigen-coated plates were incubated for 1 h with 30 μ l of 10-times-diluted mouse ascites fluids containing mAb. Every experiment included titrations of (a) immune sera after incubation of the plates with mAb against homologous CS proteins, (b) immune sera after incubation with unrelated mAb, and (c) normal monkey or human sera after incubation with an unrelated mAb. The counts obtained in group c represent background and were subtracted from those obtained in groups a and b.

Sucrose Gradient Ultracentrifugation. 50 μ l of extract obtained from 2.5×10^6 parasites was incubated with 50 μ l of a solution containing 2% sodium dodecyl sulfate (SDS) and 6 M urea.

After 1 h incubation at room temperature, 10 μ g of molecular weight markers was added (mouse IgG, BSA, ovalbumin [OVA], and soybean trypsin inhibitor [STI]). The mixture (0.2 ml) was placed on the top of a 5-ml tube containing a linear 5–20% sucrose gradient in PBS. The runs were performed in a Beckman L8-80 ultracentrifuge (Beckman Instruments, Inc., Fullerton, CA) with a SW-50.1 rotor (DuPont Instruments–Sorvall Biomedical Div., DuPont Co., Wilmington, DE) at 48,000 rpm for 15 h. Fractions of ~ 125 μ l were collected by perforating the bottom of the tube. Aliquots of each fraction were subject to SDS-polyacrylamide gel electrophoresis. The gels were dried and stained to determine the position of the markers. Each fraction was tested for the presence of antigen by the two assays described below. One of these (the inhibition assay) detects antigen molecules bearing single epitopes and the other (the two-site assay) detects only multivalent antigens.

Inhibition Assay to Detect the Presence of Antigen in Fractions of Sucrose Gradient. 10 μ l (5×10^4 cpm, 1–5 ng) of radiolabeled mAb (2G3 anti-CS protein of *P. knowlesi* or 2F2 anti-CS protein of *P. vivax*) was incubated with 30 μ l of each fraction from the sucrose gradient. After 1 h at room temperature, 30 μ l of each mixture was added to an antigen-coated plate, incubated for 1 h, washed, and counted. The results were expressed as the number of sporozoites present in each fraction as calculated from a standard curve obtained simultaneously with each assay.

Two-Site/One-Antibody Immunoradiometric Assay. Wells of a microtiter plate were incubated overnight at 4°C with 50 μ l of a 10 μ g/ml solution of purified mAb in PBS. The wells were washed and incubated for 60 min with PBS-BSA. 35 μ l of dilutions of sporozoite extracts was added to separate wells and the plates were incubated at room temperature for 4 h. The wells were washed with PBS-BSA and 30 μ l (1×10^6 cpm, 5–10 ng) of 125 I-labeled mAb (the same used to coat the wells) was added to each well. After 1 h of incubation at room temperature, the wells were washed, dried, and counted. The number of counts bound to control wells that had been coated with an unrelated mAb or with PBS-BSA before addition of extracts was negligible.

When fractions of the sucrose gradient were tested for the presence of antigen, 35 μ l of each fraction was added to the antibody-coated wells. The assay proceeded in the same way as described above. The results were expressed as the number of sporozoites contained in each fraction, as calculated from a standard curve obtained on the same day of the experiment.

Radiolabeling of mAb. 25 μ l (25 μ g) of purified mAb was radiolabeled with 1–2 mCi of 125 I, using Iodogen (Pierce Chemical Co., Rockford, IL) and the method suggested by the manufacturers. The free 125 I was removed by passage of the sample through Sephadex G-25 PD-10 columns (Pharmacia Fine Chemicals, Piscataway, NJ), followed by extensive dialysis against PBS. Specific activities of the radiolabeled mAb ranged between 2 and 3×10^7 cpm per microgram of protein.

Results

Topological Analysis of Antigenic Determinants on CS Proteins. Panels of mAb were used to study the arrangement of the epitopes of CS proteins within single molecules by using a competition binding assay (12–14). These were performed with five mAb to *P. vivax* derived from three different fusions, five mAb to *P. falciparum* from two fusions, and six mAb to *P. knowlesi* from a single fusion. Results are expressed as percentage inhibition of binding of each mAb by the others and calculated as described in Materials and Methods. As seen in Table I, all mAb to *P. vivax*, *P. falciparum*, and *P. knowlesi* inhibited the binding of each other to the homologous CS antigen. This inhibition was specific, because unrelated mAb had no activity. In some cases there was incomplete inhibition. For example, the binding of 125 I-2F2 anti-*P. vivax* was only partially inhibited in the presence of an excess of the other antibodies. However, an excess of cold 2F2 in the incubation medium inhibited 100% of the binding of all radiolabeled antibodies. Similarly, an excess of 8E11 inhibited only 60–80% of the

TABLE I
Evidence for the Presence of an Immunodominant Region on CS Proteins

Cold inhibitor (mAb)	Percent inhibition of binding to antigen of the radiolabeled mAb					
<i>P. vivax</i>	3D10	5D9	3C1	4E8	2F2	
3D10	100	102	99	101	59	
5D9	83	100	98	101	65	
3C1	113	103	100	102	67	
4E8	105	100	99	100	60	
2F2	100	101	100	116	100	
Control mAb	8	-6	-11	3	6	
PBS-BSA	0	0	0	0	0	
<i>P. knowlesi</i>	5H8	2G3	8B8	8A8	8E11	6B8
5H8	100	99	101	115	105	121
2G3	96	100	87	78	106	103
8B8	95	94	100	106	107	125
8A8	86	83	94	100	105	128
8E11	65	59	86	70	100	105
6B8	88	85	91	95	104	100
Control mAb	6	0.4	0.5	8	0	0
PBS-BSA	0	0	0	0	0	0
<i>P. falciparum</i>	3D6	2E7	1E9	2C11	2A10	
3D6	100	98	99	95	75	
2E7	98	100	102	99	90	
1E9	100	103	100	100	99	
2C11	104	100	99	100	100	
2A10	110	102	106	100	100	
Control mAb	2	0	-4	8	0	
PBS-BSA	0	0	0	0	0	

binding of several antibodies to *P. knowlesi*, but all antibodies inhibited 100% of the binding of radiolabeled 8E11 to the antigen. Identical results were obtained when four anti-*P. berghei* mAb were subjected to this analysis (not shown).

Competitive Assay Between mAb and Anti-Sporozoite Immune Sera. The experiments described below were performed to determine whether polyclonal human antibodies to sporozoites of *P. falciparum* and *P. vivax*, and monkey antibodies to *P. knowlesi* recognize the same region of the CS molecule as that seen by the homologous mAb. The immune sera were obtained from humans or monkeys vaccinated with irradiated sporozoites and shown to be fully protected when challenged with viable parasites. Wells of plates coated with crude extracts of sporozoites were first incubated with ascites fluids containing mAb to CS proteins. Then immune sera at different dilutions were added to the wells. The presence of polyclonal antibody bound to the immobilized antigen was demonstrated by the specific binding of ^{125}I -labeled, affinity-purified rabbit anti-monkey or rabbit anti-human Ig. As shown in Fig. 1, all polyclonal antisera contained antibodies that bound to the solid-phase-associated antigen, and in each case a single mAb inhibited 70–95% of the binding of the polyclonal antibodies to the wells. It appears, therefore, that in the vaccinated and protected hosts, most

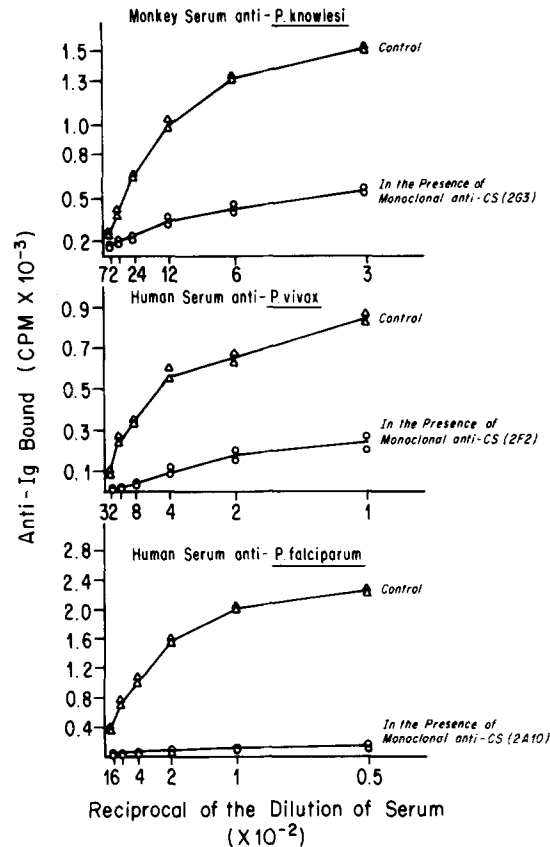


FIG. 1. Inhibitory effect of mAb to CS proteins on the binding of polyclonal antibodies to sporozoite extracts. The experiments represented above were performed in two steps. First, serial dilutions of polyclonal antisera were incubated with a crude extract of sporozoites in the presence or absence of a mAb. Then the amounts of polyclonal Ig that bound to the solid-phase-associated antigen were measured using an excess of radiolabeled, affinity-purified anti-Ig of the appropriate specificity. As shown, the mAb inhibited 70–95% of the binding of the polyclonal antibody to antigen.

circulating antibodies are directed against CS proteins, and that the mAb and most of the polyclonal antibodies recognize the same epitope.

Demonstration That the CS Proteins Contain Two or More Identical Epitopes. This was shown using a two-site immunoradiometric assay. In this type of assay the concentration of antigen is usually measured by means of two antibodies of different specificities. The first antibody, bound to a solid support, serves to isolate and concentrate the antigen. The concentration of bound antigen is then obtained by adding an excess of a second labeled antibody directed against the same antigen but recognizing a different epitope. In the experiments shown in Fig. 2 we performed two-site assays to detect and measure CS proteins in extracts of parasites, but we used a single mAb both in the solid phase and as a radiolabeled developing reagent. We reasoned that use of a single mAb would be equally effective if the antigen contained two identical and topographically separated epitopes. We performed antigen titrations with this two-site/one-antibody assay using extracts of several species of sporozoites. The results

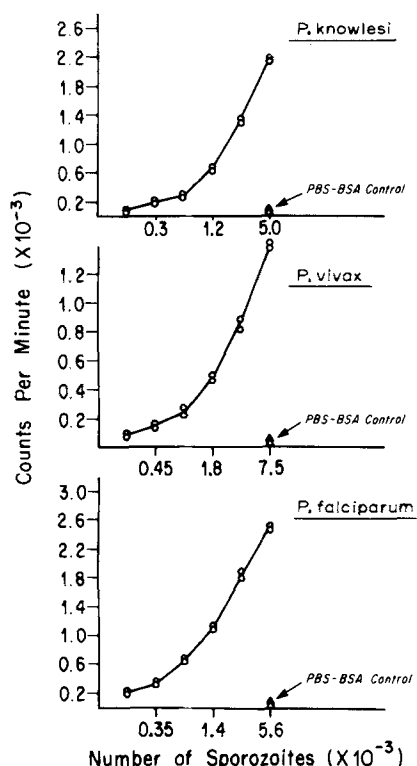


FIG. 2. Two-site/one-antibody immunoradiometric assays for detection of sporozoites. Sporozoite extracts were incubated in wells of microtiter plates coated with a mAb to a CS protein. After washing, the amount of antigen bound to the solid phase was measured by adding to the wells an excess of the same mAb radiolabeled with ^{125}I .

showed that this assay not only detected the antigen specifically, but also that its sensitivity was identical to that of the two-site/two-antibody assay described elsewhere (15); that is, positive results were obtained when extracts contained between 5×10^3 and 10^4 sporozoites/ml. Identical results to those of Fig. 2 were obtained when the diluent used in the two-site/one-antibody assay contained 0.5% NP-40.

To determine the molecular weights of the CS proteins that contain the repeated epitopes, solubilized sporozoites were obtained as described in Materials and Methods and then mixed with equal volumes of 2% SDS and 6 M urea. The mixture was overlaid onto a preformed sucrose gradient and subjected to ultracentrifugation. The presence of CS proteins in gradient fractions was determined by means of two different assays; that is, by the two-site/one-antibody immunoradiometric assay, which depends on the multivalency of the antigen, and by the inhibition of binding of radiolabeled antibody to antigen-coated plates, which can detect monovalent antigens. To facilitate comparison of the results of the two assays, the amounts of antigen detected were expressed in sporozoite equivalents, as calculated from standard curves performed on the same day as the experiment.

As seen in Figs. 3A and 4A, the CS protein of *P. vivax* and *P. knowlesi* was detected with the two-site assay in a single peak between BSA and OVA. This position is the expected one for monomers of the CS proteins (and their intracellular precursors)

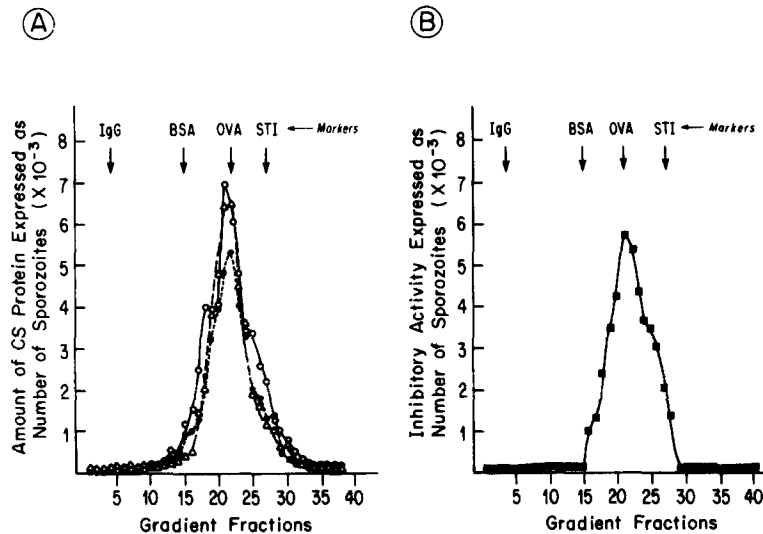


FIG. 3. Detection of CS protein in *P. vivax* extracts subjected to sucrose gradient ultracentrifugation. Extracts of *P. vivax* in 3 M urea and 1% SDS were subjected to ultracentrifugation in linear 5–20% sucrose gradients. Fractions from the gradient were analyzed by two assays. (A) The two-site/one-antibody immunoradiometric assays detect bivalent or polyvalent antigens. These assays were performed with different mAb (2F2, Δ ; 5D9, \circ ; 3C1, \bullet) with identical results. (B) The inhibitory activity of fluid-phase antigen on the binding of a radiolabeled mAb (2F2) to solid-phase-associated antigen was measured. This assay can detect monovalent antigen. Both assays detected antigen sedimenting as a single peak between the OVA and BSA markers.

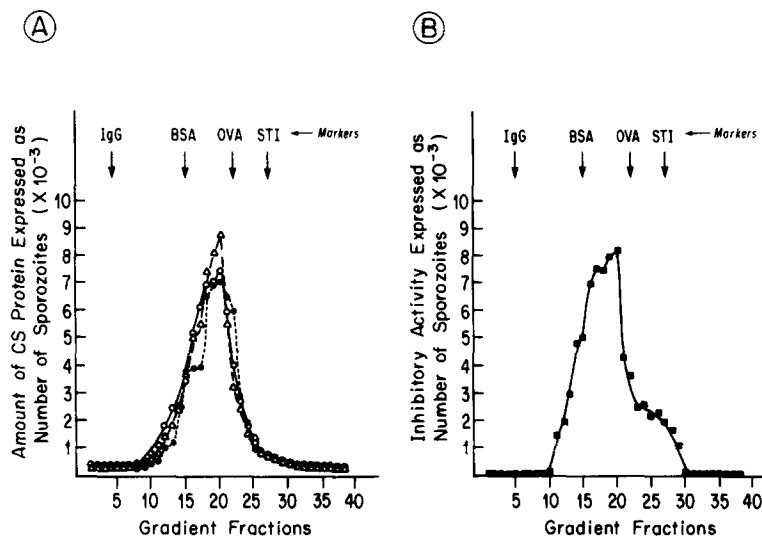


FIG. 4. Detection of *P. knowlesi* CS protein in parasite extracts subjected to sucrose gradient ultracentrifugation. Extracts of *P. knowlesi* were analyzed exactly as described in the legend of Fig. 3. Assays were performed with 2G3 (Δ), 5H8 (\circ), and 8E11 (\bullet). The CS protein sedimented as a single peak between the OVA and BSA markers.

that have molecular weights between 40,000 and 60,000 (6). We calculated that the antigen recovered in this peak represented >90% of the amount loaded onto the gradient. Similar results were obtained when the fractions were tested by the inhibition

assay (Figs. 3 *B* and 4 *B*).

Identical results were obtained when CS proteins from *P. falciparum* were subjected to this analysis (not shown). It should be pointed out that two-site/one-antibody immunoradiometric assays of gradient fractions were performed simultaneously with several individual mAb. As also shown in Figs. 3 *A* and 4 *A*, the antigenic profiles obtained with all homologous mAb were superimposable. These findings strongly suggest that each monomer is multivalent with respect to epitope expression.

Discussion

The purpose of the experiments described in this paper was to study the number of independent antigenic sites of CS proteins. Our initial approach was to use a competitive binding assay to determine the number of nonoverlapping sites recognized by a panel of mAb. The underlying principle of this assay is that the binding of a radiolabeled antibody to an antigen will be prevented only by other antibodies that recognize the same, or topographically related, sites. Of course, this assumption will be considerably strengthened if the inhibitory effects of the ligands are reciprocal (12–14). As shown in Table I, all mAb to CS proteins of *P. falciparum*, *P. vivax*, and *P. knowlesi* interacted competitively with the respective antigens. The inhibitory effects were quite strong, ranging from 60 to 100%. In a few instances the competition was asymmetrical. This is probably a reflection of the difference in avidity between the antibodies, because in these reactions, the ligand with higher avidity will be favored. Similar effects have been previously described in other systems (12).

The simplest interpretation of these results is that all mAb to CS proteins recognize a single region of the protein, which is immunodominant. This hypothesis is supported by the experiments shown in Fig. 1, which demonstrate that single mAb to the CS protein of *P. vivax*, *P. falciparum*, or *P. knowlesi* inhibited 70–95% of the binding of polyclonal antibodies to the respective antigens. It should be pointed out that the polyclonal antisera had been prepared by vaccination of humans and monkeys with irradiated intact sporozoites and that the assays were performed with crude extracts of sporozoites as a source of antigen. Therefore, it appears that not only are the CS proteins the most immunogenic constituents of sporozoites, but also that the epitope recognized by the immune system of monkeys and humans is identical to that recognized by the mAb.

The detection in CS proteins, whose molecular weights vary between 40,000 and 60,000, of a single immunoreactive area, was unexpected. For example, molecules as small as myoglobin (16,900 mol wt) have five different immunoreactive areas and bind four antibody molecules simultaneously (16). Also, using the competitive binding assay, two epitopes have been found on both envelope proteins of leukemia virus (70,000 and 15,000 mol wt) (12).

The explanation for the high immunogenicity of a single region of the CS proteins is not known. The finding that these molecules contain recurrent epitopes may be relevant because the multiplicity within CS proteins of possible recognition sites by cells of the immune system could increase their antigenicity. The presence of several identical or similar epitopes within CS proteins was demonstrated by their ability to bind simultaneously two or more molecules of the same mAb (Fig. 2). To exclude the

possibility that the apparent multivalence is artifactual and a consequence of aggregation of the antigen, parasite extracts were treated with SDS-urea and subjected to ultracentrifugation in sucrose gradients. Analysis of gradient fractions by two methods showed that a multivalent antigen sedimented between BSA and OVA, as expected for monomers of CS proteins (Figs. 3 and 4).

Another important observation was that every mAb tested detected a recurrent epitope (Figs. 3A and 4A). This finding strongly suggests that all antibodies recognize a unique structure of the molecules present in the immunodominant region. In agreement with these observations, a cDNA clone from the CS protein of *P. knowlesi* has been recently isolated, and the sequence encoded by this clone was expressed in a β -lactamase fusion protein of the plasmid pBR322. The fusion protein was detected by the two-site/one-antibody assay. Preliminary DNA sequencing data indicate that the expressed fragment of CS protein contains multiple repeats of a sequence of amino acids that react with the mAb (17).

Whatever the reason for the unique immunologic properties of CS proteins, the present findings, taken together with previous observations, have implications for the development of a malaria vaccine. In the first place, it is clear that CS proteins are potent immunogens. Every mAb produced to date in our laboratory against the surface membrane of sporozoites has been shown to be directed against a CS protein. Also, the sera of volunteers vaccinated with sporozoites of *P. vivax* or *P. falciparum* (this paper and reference 5), or the serum of adults living in endemic areas (18), contain antibodies to CS proteins.

Perhaps of even greater significance is the demonstration that a single recurrent epitope appears to contain most of the immunogenic activity of CS proteins. Because the binding of Fab fragments of mAb to this structure neutralizes the infectivity of sporozoites, it is possible that a small portion of the CS molecule represented in this epitope could be used to produce protective immunity in the host.

Summary

We have used panels of monoclonal antibodies to circumsporozoite (CS) proteins of *Plasmodium falciparum*, *P. vivax*, and *P. knowlesi* to determine the number of topographically independent epitopes of these antigens. The results of competition binding assays indicated that single regions of the CS molecules were recognized by the homologous monoclonal antibodies. Competition binding assays were also used to study the specificity of antibodies contained in the sera of humans and monkeys that had developed sterile immunity after immunization with irradiated, intact sporozoites. We found that single monoclonal antibodies inhibited 70–95% of the specific binding of the polyclonal antibodies to crude extracts of sporozoites. It appears, therefore, that CS proteins are among the most immunogenic constituents of sporozoites, and that a single region of these molecules contains most of the immunogenic activity.

An additional finding was that the immunodominant region of CS molecules is multivalent with regard to the expression of a single epitope. This was demonstrated by the ability of monomers of CS proteins to bind simultaneously two or more molecules of the same monoclonal antibody.

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