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Plasmodium berghei Sporozoite Invasion Is Blocked In Vitro by Sporozoite-Immobilizing Antibodies

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A monoclonal antibody directed against the circumsporozoite protein on the surface of *Plasmodium berghei* sporozoites inhibited sporozoite motility in vitro. These immobilized sporozoites could adhere to but not invade target cultured cells. Other sporozoite-immobilizing agents also inhibited sporozoite invasion into cultured cells and did not prevent sporozoite adherence. These results indicate that sporozoite invasiveness is associated with sporozoite motility. Thus, the immobilizing effect of this antibody could explain its functional activity against sporozoite invasion in vivo.

Malaria is initiated when female anopheline mosquitoes inject *Plasmodium* sporozoites into the circulatory system of their host, while ingesting blood. Since protective immunity is mediated by antibodies directed against the circumsporozoite (CS) protein on the sporozoite surface (35), vaccination with CS proteins could be a practical way of protecting humans against malaria. The amino acid sequence of the CS protein of *Plasmodium falciparum* sporozoites has been determined (6, 8), and an attempt to vaccinate with synthesized portions of this functional antigen appears possible (2, 36, 39).

However, a major obstacle to an antisporozoite vaccine is the speed with which sporozoites evade the immune response. Sporozoites can penetrate hepatocytes within minutes after inoculation into the host (26); once inside a liver cell, they are no longer susceptible to antibody assault (9, 29). This remains the major problem with antisporozoite vaccines; some immunized animals may develop anti-CS protein antibodies but not be protected against challenge with viable sporozoites (3–5, 10, 18, 22, 23, 33; D. Chen, Ph.D. thesis, New York University School of Medicine, New York, 1974). Thus, it is crucial to understand how anti-CS protein antibodies act against sporozoites during their fleeting moment of vulnerability.

Anti-CS protein monoclonal antibodies (MAbs) prevent the entry of sporozoites into normally susceptible cells in vitro (13, 16). Furthermore, the CS protein appears to become associated with the target cell membrane during the penetration of sporozoites in vitro (J. M. Ramsey, R. L. Beaudoin, and M. R. Hollingdale, Proc. VI Int. Congr. Protozool., p. 305, 1981). Accordingly, Hollingdale et al. (13) reasonably suggested that the CS protein is intimately involved in sporozoite entry into cells and that immunity to sporozoites in vivo appears to be mediated by the binding of antibody to the protein, thereby inhibiting the interaction of the sporozoite with its target hepatic cell.

Polyclonal antisporozoite antibodies induce a morphological alteration of sporozoites, termed the CS precipitation (CSP) reaction (32) and immobilize actively motile sporozoites in vitro (30). If anti-CS protein MAbs also immobilize sporozoites, this in itself could explain the failure of such sporozoites to reach and actively penetrate host cells. Therefore, we tested whether such an MAb was capable of immobilizing sporozoites. We also assessed the relationship between sporozoite motility and the ability of sporozoites to invade cells in vitro, with an anti-CS protein MAb and other sporozoite-immobilizing agents. Our findings demonstrate that the invasive ability of sporozoites is directly associated with their motility and that sporozoites are rapidly immobilized by an MAb directed against the repeating immunodominant epitope of the CS protein.

MATERIALS AND METHODS

The methods for maintaining the *Plasmodium berghei* (NK65 strain)-mosquito-hamster system of rodent malaria were previously described (31). Sporozoites were obtained from the salivary glands of infected *Anopheles stephensi* mosquitoes 18 to 19 days after they had fed on infective hamster blood.

Production of MAb H4 and Fab fragments. A/J mice (8 to 10 weeks old) were inoculated intravenously with 300,000 gamma-irradiated (8 krad) sporozoites, followed by four booster inoculations of 100,000 gamma-irradiated sporozoites at 4-day intervals. The mice were bled 3 days after the final booster injection, at which time the serum showed a positive CSP reaction (32). Immune splenocytes were separated, concentrated, and fused with P3U1 plasmacytoma cells (15, 21). Supernatant fluids from cultures of potential hybridomas were screened by indirect immunofluorescence of the sporozoite surface (19); hybridomas from cultures producing the most intense fluorescence were cloned by limiting dilution. Hybridomas were injected intraperitoneally into $CD2F_1$ mice, and ascites fluid was subsequently collected. The MAb (which we designate as H4) from 10 ml of ascites fluid was purified by ammonium sulfate precipitation (45% saturated ammonium sulfate), extended dialysis, and gel filtration through Sephadex G-200. The dialysis and elution buffers comprised phosphate-buffered saline containing 0.2% sodium azide. Fab fragments of H4 were prepared by papain digestion (20, 21) for 18 h, and the reaction was terminated by the addition of excess iodoacetamide. The papain digest was dialyzed and subjected to Sephadex G-200 gel filtration. H4 and its Fab fragments were concentrated, dialyzed against distilled water, and stored at −70°C until used. Purified H4 and its Fab fragments were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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under reducing conditions in a 10% polyacrylamide gel, and the gel was stained with Coomassie blue. Immunoradiometric binding assays between H4 and 3D11, the original anti-*P. berghei* CS protein (Pb44) MAb described by Yoshida et al. (35), were performed as previously described (37).

Sporozoite-immobilizing agents. Since the purpose of this study was to determine the relationship between sporozoite motility and invasiveness, the motility and invasion assays were done under identical in vitro conditions. A number of the immobilizing agents tested (human α- and γ-globulins from nonimmune serum [Miles Laboratories, Inc.] and H4 and Fab fragments against the *P. berghei* sporozoite surface) have no apparent detrimental effects on the host cell. Thus, these reagents could be added directly into the host-parasite invasion culture medium. However, cytochalasins B and E are known to disrupt microfilaments of eucaryotes and interfere with microfilament-associated processes, such as phagocytosis by host cells; it would thus be difficult to determine whether a drug-induced reduction in invasion was attributable to an effect on host cells, sporozoites, or both. Accordingly, when cytochalasin B or E was tested, sporozoites were pretreated with the drugs, rinsed, and added to cell cultures in medium devoid of these drugs.

The globulins, which were added at higher-than-physiological concentrations, immobilize sporozoites without killing them at these concentrations (30). In one series of experiments, sporozoites were pretreated with α-globulin for 30 min at room temperature, rinsed, and subjected to the motility and invasion assays.

Control preparations of sporozoites normally received no additives. However, when MAb H4 and Fab fragments were tested, control preparations received an equivalent concentration of an immunoglobulin G1 MAb directed against the homologous CS protein epitope on *Plasmodium knowlesi* sporozoites (kindly supplied by Alan Cochrane, New York University Medical Center).

Sporozoite motility assay. Under defined conditions, *P. berghei* sporozoites are motile in vitro (30); that is, they undergo circular gliding or attached waving motility on the surface of a microscope slide or cover slip. The sporozoite motility assay was performed and quantitated as previously described (30) with slight modifications necessary to duplicate the conditions of the in vitro invasion assay. Sporozoites were suspended in Eagle minimum essential medium, 10% fetal bovine serum, penicillin (50 U/ml), and streptomycin (5 μg/ml) (MEM^+) and kept at 4°C until used. The sporozoites were then mixed with appropriate concentrations of candidate-immobilizing agents dissolved in MEM^+ and incubated for 30 min at 22°C. The control or treated sporozoite preparation (5 μl of each) was pipetted onto a microscope slide, covered with a cover slip, and allowed to stand for 10 min to enable the sporozoites to settle and attain ambient temperature. The slides were examined under a phase-contrast microscope whose stage was warmed to 37°C, and the percent motility of treated sporozoites was compared with that of the control preparations. A given sporozoite was deemed motile if it underwent circular gliding or attached waving motility within 30 s of observation. The slides were coded blindly by one investigator and read by another.

When cytochalasin B or E was tested, sporozoites were incubated in the drug (dissolved in a small volume of dimethyl sulfoxide and diluted with MEM^+) for 30 min at 22°C, then diluted 10-fold with MEM^+, and incubated at 37°C for 30 min before the percent motility was determined.

In vitro invasion assay. The in vitro invasion assay was performed as described by Hollingdale et al. (12) with slight modifications. Human embryonic lung fibroblasts (WI38; American Type Culture Collection, Rockville, Md.) and human hepatoma (HepG2) cells (kindly supplied by Barbara Knowles of The Wistar Institute, Philadelphia, Pennsylvania) served as target host cells. The cells were grown to confluence on 12-mm-diameter glass cover slips set in 24-well tissue culture plates (Costar, Cambridge, Mass.). To initiate the invasion studies, HepG2 cells were incubated in 0.3 ml of MEM^+ with or without immobilizing agents (except cytochalasin B or E); WI38 cells were incubated in medium NCTC 135 (GIBCO Laboratories, Grand Island, N.Y.), 10% fetal bovine serum, penicillin (50 U/ml), and streptomycin (5 μg/ml; $NCTC\ 135^+$) with or without immobilizing agents. Sporozoites (30,000 in 30 μl) were pipetted into each culture well, and the wells were gently agitated to randomize the distribution of sporozoites. The sporozoites were allowed to invade host cells for 2 h at 37°C and rinsed thoroughly with two changes of MEM^+ or medium $NCTC\ 135^+$, followed by two more rinses with Dulbecco phosphate-buffered saline (pH 7.2). Fluids were removed by vacuum aspiration. Preparations were fixed for 10 min in ice-cold methanol.

To test the effects of cytochalasins B and E on sporozoite invasion of host cells, sporozoites were incubated in either drug (diluted in the appropriate medium) for 30 min at room temperature. Sporozoites (30,000 in 30 μl) were then pipetted into wells of cultured cells containing 0.3 ml culture medium and allowed to incubate for 2 h at 37°C as described above.

Immunoperoxidase staining. H4 (50 μg/ml in phosphate-buffered saline containing 1% bovine serum albumin) served as the first antibody in the immunoperoxidase antibody test (12). All rinses after the primary antibody treatment were with phosphate-buffered saline containing 1% bovine serum albumin. Peroxidase-conjugated rabbit immunoglobulins against mouse immunoglobulins were produced by Dako Immunoglobulins and distributed by Accurate Chemicals, Westbury, N.Y. The dried cover slips were mounted on microscope slides with Permount, blindly coded by one investigator, and read by another investigator with bright-field optics. Morphological criteria for distinguishing invasions from mere adherences are defined in the figure legends. Sporozoites that had not invaded cultured cells but were present on the surface of these cells after the four rinses prior to methanol fixation were considered adherent to host cells.

RESULTS

Verification of MAb H4 and Fab fragments. MAb H4, which is secreted by the hybridoma that we isolated, is directed against the surface of *P. berghei* sporozoites; purified H4 gave a striking sporozoite surface indirect immunofluorescence test and induced an intense CSP reaction from sporozoites but only from those suspended in medium containing serum or albumin. Immunoradiometric binding assays between H4 and 3D11 demonstrated that H4 recognized the same repetitive immunodominant epitope as 3D11. Ouchterlony double diffusion precipitin reactions between culture supernatant H4 and a series of rabbit anti-mouse immunoglobulin subclasses indicate that H4 is of the immunoglobulin G1 subclass. A sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel of the Sephadex G-200 peak containing H4 and the papain digest (Fab fragments) of that peak electrophoresed under reducing condi-

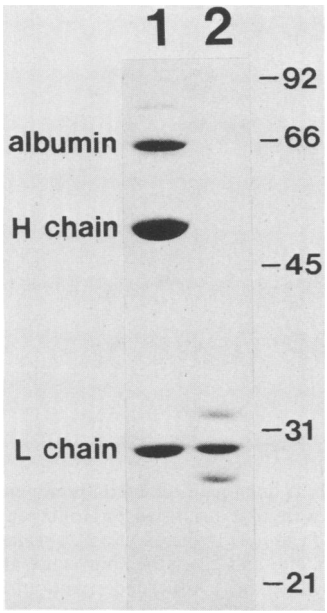

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel of the Sephadex G-200 peak containing MAb H4 (lane 1, 40 μg) and the papain digest (Fab fragments) of that peak (lane 2, 25 μg) electrophoresed under reducing conditions. Lane 1 contains heavy (H)- and light (L)-chain fragments of the immunoglobulin G1 H4, albumin, and unidentified minor proteins. Lane 2 contains the light chain of H4 and two other bands. Note that the papain digest (lane 2) is not contaminated with intact H4; i.e., heavy chains are absent. The locations of molecular weight markers, expressed in thousands, are indicated in the rightmost column of the figure.

tions is shown in Fig. 1. The papain digest was not contaminated with heavy chains, indicating that no intact H4 molecules were detectable in the papain digest. Furthermore, even though the surface of fixed sporozoites demonstrated a positive indirect immunofluorescence test after incubation with the papain digest and intact H4, viable sporozoites failed to produce the CSP reaction when incubated with the papain digest as previously demonstrated (21).

Morphological criteria distinguishing sporozoite invasions from adherences. The morphological criteria distinguishing sporozoite invasions into cultured cells from mere adherences are shown in Fig. 2A through C. The immunoperoxidase reaction product surrounding sporozoites that have invaded host cells in vitro is shown in Fig. 2A. The widely diffused reaction product around such invasive sporozoites is distinctly different from that seen around sporozoites that have merely adhered to the host cell surface without invading (Fig. 2B), as was seen after treatment of sporozoites with immobilizing agents. Sporozoites exposed to anti-CS protein MAb (H4) produced prominent CSP reactions and adhered to the surface of host cells without invading (Fig. 2C).

Relationship between sporozoite motility and invasiveness in vitro. The mean percentage of control sporozoites that invaded HepG2 cells was 10.5, with a range of 2.8 to 23.5 between experiments. The mean percentage of control sporozoites that invaded WI38 cells was 5.3, with an experimental range between 3.3 and 8.4. The mean percentage of control sporozoites that demonstrated motility was 79 (standard deviation, 11); the experimental range was 64 to 100.

The effects of various agents and treatments on sporozoite motility and invasiveness in vitro are shown in Table 1. In each case, there was a direct relationship between the inhibition of sporozoite motility and inhibition of invasion; the correlation coefficient, r, between sporozoite motility and invasiveness was >0.94. H4 (200 μg/ml), Fab fragments of H4 (500 μg/ml), heat inactivation, and cold treatment of sporozoites virtually abolished both sporozoite motility and invasiveness. Human α-globulin at 2.5 and 5%, 3% human γ-globulin, and pretreatment with 10^{-4} M cytochalasin B or 10^{-5} M cytochalasin E virtually abolished sporozoite motility and substantially reduced the invasion of sporozoites into host cells. Pretreatment of sporozoites with 5% α-globulin, followed by rinsing this reagent from sporozoites and inoculating these sporozoites into the motility and invasion assays, did not prevent sporozoites from invading cultured cells or inhibit motility; i.e., the inhibitory effects were reversible. Pretreatment of sporozoites with a lower concentration of cytochalasin B (10^{-5}M) was relatively ineffective in blocking sporozoite motility and invasiveness. Gamma irradiation, which has no effect on the invasion of HepG2-A16 cells (27), had no inhibitory effect on sporozoite motility and invasion into WI38 and HepG2 cells.

DISCUSSION

Hollingdale et al. (13) previously demonstrated that the MAb (3D11) that recognizes Pb44, the CS protein on the surface of *P. berghei* sporozoites, inhibits the invasion of these sporozoites into host cells in vitro. Homologous anti-*P. knowlesi* sporozoite MAb did not inhibit *P. berghei* sporozoite invasion into target cells. They concluded that "the immunity to sporozoites in mice appears to be mediated by the binding of antibody to Pb44 on the sporozoite surface inhibiting its interaction with its target hepatic cell". However, the data presented here suggest that this antibody exercises its protective effect by interfering with the sporozoite motility required for active invasion of host cells. Earlier studies suggested that motility and invasiveness of sporozoans are related (7, 14, 24, 30, 34; reviewed in reference 28). We have now shown that anti-CS protein MAbs and their Fab fragments directed against a single epitope on the CS protein are capable of both immobilizing sporozoites and inhibiting their invasiveness. This strongly suggests that these two specifically defined functional effects of antisporozoite humoral immunity, namely, sporozoite immobilization and inhibition of sporozoite invasion, are causally related.

We used the criteria of Hollingdale et al. (12) to distinguish between sporozoite adherence to and invasion of cultured cells, as observed in our own preparations (Fig. 2A through C). The basis of this assay resides in the ability to correlate light-microscopic observations of immunoperoxidase-stained preparations with fine structural studies of parallel sporozoite-host cell preparations. Electron microscopy of control preparations (without inhibitory agents) has demonstrated incontrovertible evidence of invasion of cultured host cells (1). Similarly, cytochalasin-treated sporozoites (which show little invasion of host cells with the light-microscopy immunoperoxidase antibody test) have been shown to adhere to but not invade at the electron-microscopic level (M. R. Hollingdale, personal communication).

Our findings on the invasion rate of *P. berghei* sporozoites into human hepatoma (HepG2) cells and human embryonic lung fibroblasts (WI38) are similar to those first reported by Hollingdale et al. (11, 12). We observed that 10.5% of the inoculated sporozoites invaded control cultures of HepG2

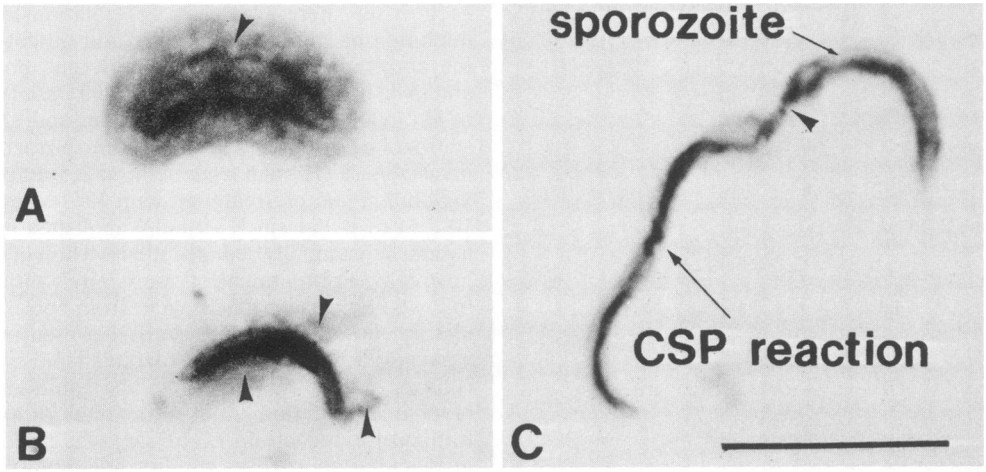

FIG. 2. *P. berghei* sporozoite invasion into (A) versus adherence to (B and C) target cells as demonstrated by immunoperoxidase antibody test. Criteria for distinguishing sporozoite invasion from adherence are outlined below and were first described by Hollingdale et al. (12). The photographic negatives were enlarged to the same final magnification; the bar represents 10 μm. (A) Invasion of a *P. berghei* sporozoite into a WI38 cell. The immunoperoxidase reaction product conforms to the shape of the sporozoite and gives the sporozoite an enlarged image (aura) due to the presence of reaction product within the parasitophorous vacuole (arrowhead). (B) Adherence (without invasion) of a *P. berghei* sporozoite to a WI38 cell in culture medium containing 2.5% human α-globulin, a sporozoite-immobilizing agent. This sporozoite has the typical size of *P. berghei* sporozoites. Some CS protein reaction product radiates from the sporozoite surface (arrowheads) but is not contained within a parasitophorous vacuole. (C) Adherence of a *P. berghei* sporozoite and its CSP reaction product to a WI38 cell in culture medium containing MAb H4. The serpentine CSP reaction trails from one end of the sporozoite (arrowhead). Note the apparent size (width) difference between the surface-adherent sporozoites in Fig. 2B and C and a sporozoite (with its aura) that has invaded a target cell (Fig. 2A).

cells; Hollingdale et al. (12) reported an 8% invasion rate into a cloned line (HepG2-A16 [25]) of these cells. It is noteworthy that the widely available cell line HepG2 can be used as effectively as the cloned line HepG2-A16, which has not been available for distribution. The present study confirms the original findings of Hollingdale et al. (12) that sporozoites invade HepG2 cells more effectively than they invade WI38 cells.

To test further our hypothesis that the immobilizing effects of anti-CS protein antibody could account for its ability to block sporozoite invasion, we assessed the invasion-blocking effects of a series of agents known to immobilize sporozoites (28, 30). Our results confirm a relationship between sporozoite motility and invasion and further support our hypothesis that the functional in vitro effects of anti-CS protein antibody are due to sporozoite immobiliza-

TABLE 1. Effects of sporozoite-immobilizing agents on invasiveness of *P. berghei* sporozoites in vitro

Pretreatment	Addition to medium	% (mean ± SD) of control for:		
		Motility[a]	Invasion[b] of WI38 cells	Invasion[b] of HepG2 cells
None (Control)	None	100	100	100
None	None, invasion at 4°C	0[c]	ND[d]	3.8 ± 3.1
None	MAB H4 (200 μg/ml)	0	0	0
None	Fab of H4 (500 μg/ml)	5.1 ± 7.2	0	0.1 ± 0.2
None	Fab of H4 (200 μg/ml)	22.8 ± 25.0	ND	ND
None	2.5% human α-globulin	1.3 ± 2.5	31.0 ± 2.8	26.1 ± 9.9
None	5.0% human α-globulin	2.5 ± 5.1	19.5 ± 2.1	13.0 ± 4.7
None	3.0% human γ-globulin	6.3 ± 12.7	40.5 ± 9.2	41.6 ± 17.6
10^{-4} M cytochalasin B[e]	None	0	28.5 ± 7.8	17.4 ± 6.1
10^{-5} M cytochalasin B[e]	None	73.7 ± 23.9	71.0 ± 7.1	92.2 ± 9.3
10^{-5} M cytochalasin E[e]	None	0	ND	7.8 ± 3.8
5% human α-globulin[e]	None	100	ND	95.3 ± 3.6
Heat inactivation[f]	None	0	0	0
Gamma irradiation[g]	None	98.3 ± 7.1	111.0 ± 1.4	120.0 ± 11.0

[a] At least 25 sporozoites were counted per preparation. The data indicate the percentage of sporozoites demonstrating active circular gliding or attached waving motility as compared with the controls.

[b] Assessment of invasion by immunoperoxidase staining of 2-h invasion preparations (12). Counts of positive invasions were determined from at least 105 fields of 81 μm^2 each from each of three replicate preparations from each experiment. The data indicate the percentage of sporozoites that have successfully invaded cultured cells as compared with the controls.

[c] The motility assay was performed at 4°C.

[d] ND, Not done.

[e] Sporozoites were pretreated with the agents indicated and washed of these agents prior to subjecting the sporozoites to the motility and invasion assays.

[f] Heat inactivation was at 60°C for 20 min.

[g] Gamma irradiation was to 8,000 rads.

tion. Furthermore, inhibition of sporozoite motility and invasiveness by chemical agents (Fig. 2B) and MAb H4 (Fig. 2C) and its Fab fragments did not prevent sporozoite interaction with target cells; they fail to penetrate despite successful adherence.

These effects of anti-CS protein antibody on sporozoite invasion in vitro presumably occur in vivo. Before invading hepatocytes, sporozoites must first cross the sinusoidal barrier separating hepatocytes from the circulation; immobilization of circulating sporozoites by antibody in an immune animal would probably prevent sporozoites from reaching hepatocytes. Whether the sporozoite penetrates the sinusoid through endothelial cells (26) or Kupffer cells (17), it is likely that either route requires active locomotion by the sporozoite (see reference 28 for a review). Sporozoite recognition of a putative hepatocyte receptor (12, 13, 27, 38; J. M. Ramsey et al., Proc. VI Int. Congr. Protozool., 1981) would be of secondary importance, since the sporozoite does not encounter the hepatocyte as an initial target. In an immune animal, sporozoites immobilized in the plasma would not likely reach the space of Disse; even those able to enter the space of Disse and adhere to hepatocytes might still lack adequate motility to allow penetration into hepatocytes. If sporozoites are thus immobilized by antibody, any subsequent interference with an interaction between sporozoite CS protein and the surface of hepatocytes would be irrelevant to functional humoral immunity.

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