MONOVALENT FRAGMENTS (Fab) OF MONOCLONAL ANTIBODIES TO A SPOROZOITE SURFACE ANTIGEN (Pb44) PROTECT MICE AGAINST MALARIAL INFECTION*

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Repeated inoculation of mice, monkeys, and humans with x-irradiated sporozoites can lead to sterile immunity against malarial infection (1, 2). Vaccinated animals have serum antibodies capable of neutralizing sporozoites (3) and of reacting with their surface membranes (4, 5). Exposure of humans to infected mosquitoes in endemic areas leads to the development of relatively high serum titers of antisporozoite antibodies (6). Upon incubation with immune serum, viable sporozoites develop at their posterior end a thread-like precipitate which increases progressively in length (circumsporozoite precipitation [CSP][1] reaction [7]).

To clarify the nature of the antibody-parasite interaction and its relation to protective immunity, we recently used the technique of Köhler and Milstein (8) to obtain hybrid cells between a plasmacytoma line and spleen cells from mice that produced antibodies to surface antigens of *Plasmodium berghei.* One clone of hybrid cells produced antibodies against a protein with an apparent 44,000 mol wt (Pb44), which envelops the surface membrane of sporozoites. In vitro incubation of the parasites with these antibodies abolished their infectivity (9).

In this paper, we study the effects of this antibody and of its fragments on sporozoites in vitro and their protective effects in vivo against malarial infection.

Materials and Methods

Media and Reagents. Phosphate-buffered saline (PBS), Dulbecco's modified Eagle's medium (DMEM), antibiotic-antimycotic solution, horse serum, and fetal calf serum, were obtained from Grand Island Biological Co., Grand Island, N. Y. Medium 199 and RPMI-1640 were obtained from Microbiological Associates, Walkersville, Md. Hypoxanthine (H), aminopterin (A), thymidine (T), Hepes (sodium salt), and dimethyl sulfoxide were obtained from Sigma Chemical Co., St. Louis, Mo. Polyethylene glycol-1000 was obtained from the J. T. Baker Chemical Co; Phillipsburg, Pa. Pristane was obtained from Aldrich Chemical Co., Inc.,

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[1] *Abbreviations used in this paper:* A, aminopterin; CSP, circumsporozoite precipitation; DMEM, Dulbecco's modified Eagle's medium; DMEM-HT complete medium, DMEM that contained 10% fetal calf serum, 1% antibiotic-antimycotic solution, 3×10^{-4} M thymidine, and 10^{-3} M hypoxanthine; H, hypoxanthine; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; Staph A, *Staphylococcus aureus,* Cowan I strain; T, thymidine.

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Milwaukee, Wis.; MOPC-21, IgG1 myeloma protein was obtained from Litton Bionetics Laboratory Products, Kensington, Md.; and agarose (Seakem; Marine Colloids, Inc., Div., FMC Corp., Rockland, Maine). DEAE-Sephadex A-50 and Sephadex G-200 were obtained from Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.

The chemicals for sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) were obtained from the following sources: *N,N'*-methylene-bis-acrylamide, acrylamide, *N,N,N',N'*-tetramethylene diamine, and ammonium persulfate from Bio-Rad Laboratories, Richmond, Calif.; X-omat R film XR5 was obtained from Eastman Kodak Co., Rochester, N. Y.

Antisera. Fluorescein-conjugated rabbit anti-mouse IgG was purchased from Meloy Laboratories Inc., Springfield, Va. A rabbit antiserum to mouse α- and κ-chains was prepared by injection of purified myeloma protein and was a gift from Dr. Michael E. Lamm, New York University School of Medicine, New York. Specific antisera to mouse IgG1 and to mouse κ-chains were purchased from Litton Bionetics Laboratory Products.

Animals. Female mice of the BALB/c and A/J strains were obtained from The Jackson Laboratory, Bar Harbor, Maine.

Purification of Sporozoites, Radiolabeling, and Preparation of Extracts. Sporozoites of *P. berghei* (NK 65 strain) were obtained from *Anopheles stephensi* mosquitoes 15–18 d after their infective blood meal (10). The purification procedure, the radiolabeling of cell membranes with ^{125}I, and the preparation of extracts are described elsewhere (9). Sporozoites of *Plasmodium yoelii nigeriensis, Plasmodium yoelii yoelii,* and *Plasmodium chabaudi,* which originated from mosquitoes fed on mice infected with isolates of the blood stage of the parasites, was kindly provided by Dr. D. Wallicker, University of Edinburgh, Edinburgh, Scotland.

CSP Reactions. Sporozoites were incubated at 37°C for 30 min in Medium 199 that contained 10% normal mouse serum or in a dilution of antibodies in the same medium. The morphological changes in the parasites were followed under a Zeiss microscope (Carl Zeiss, New York) (magnification: × 400, phase objective).

Immunization of Mice. P. berghei sporozoites were given to anesthetized BALB/c mice once a week by bite of γ-irradiated infected mosquitoes according to the method described by Vanderberg et al. (11). The animals were used after the sixth booster, when they had developed high CSP titers and were protected against sporozoite challenge.

Production of Hybridoma. We used the method described by Köhler and Milstein (8) and Pontecorvo (12) with minor modifications. The whole procedure was carried out at room temperature. Spleen cells from BALB/c mice immunized with *P. berghei* were obtained 4 d after the last booster. 1.4×10^8 washed spleen cells and 1.4×10^7 plasmacytoma cells (P3U1, kindly given to us by Dr. J. Unkeless, The Rockefeller University, New York) were used for fusion. After they were mixed, the cells were washed once by centrifugation in a conical glass tube with ~20 ml of DMEM that contained 20 mM Hepes buffer, pH 7.2, in serum-free medium (200 *g* for 10 min). The pellet was resuspended gently in 1 ml of a solution that contained 35% polyethylene glycol 1000, 5% dimethyl sulfoxide in RPMI-1640 medium. Immediately afterwards, the cells were further diluted by adding, in succession, 3 ml of DMEM in ~3 min, followed by 12 ml of DMEM with 20% horse serum in ~5 min. The tube was rotated slowly during this procedure. Subsequently, the cell suspension was centrifuged at 200 *g* for 10 min and resuspended in 50 ml of DMEM that contained 10% fetal calf serum, 1% antibiotic-antimycotic solution, 3×10^{-4} M of T_1, and 10^{-3} M of H (DMEM-HT complete medium). The suspension was distributed in aliquots of ~50 μl per well in 96-well plates (Linbro Chemical Co., Hamden, Conn.) 24 h later, another 50 μl of DMEM-HT complete medium plus 10^{-5} M A were added to each well. Supernates were tested for the presence of antibodies to sporozoites by indirect immunofluorescence.

Purification of Monoclonal Antibodies and Preparation of Fab Fragments. Ascites was induced by intraperitoneal inoculation of hybrid cells into BALB/c mice previously injected with pristane. 100 ml of ascitic fluid that contained ~2 mg/ml of antibodies to Pb44 were dialyzed against 0.01 Tris-HCl buffer, pH 8.6, that contained 0.02 M NaCl (conductivity 1.6 mS at 0°C). After centrifugation at 10,000 *g* for 30 min, the supernate was loaded onto a 2.5- × 30-cm column that contained DEAE-Sephadex A-50 equilibrated in the same buffer. The column was washed extensively with the starting buffer until the OD at 280 nm of the eluate was <0.050. A linear

NaCl gradient (to 12 mS) was then applied. The monoclonal antibodies eluted in a sharp peak in tubes with conductivities close to 5 mS. These fractions were pooled, concentrated, and subjected to molecular-sieve chromatography in Sephadex G-200. The antibody activity was associated with the material eluted in the first OD peak. About 100 mg of antibodies were recovered. The antibodies were digested with papain to prepare Fab fragments using the method described by Nisonoff (13). The digestion was stopped by addition of excess iodoacetamide and the antibody fragments were further purified by molecular-sieve chromatography on Sephadex G-200. No intact antibodies were present in the Fab preparation as shown by SDS-PAGE (Fig. 1).

Immunoprecipitation. Immunoprecipitation was performed as described by Kessler (14) using a suspension of formaldehyde-treated *Staphylococcus aureus*, Cowan I strain (Staph A) to bind immune complexes. The monoclonal antibodies to Pb44 are of the IgG1 subclass (Results). For this reason, and to enhance the binding of complexes to Staph A, all reactions and washings were carried out in Tris-buffer at pH 8.6 (15). Eluates from the bacteria in sample buffer that contained 2% SDS, 10% glycerol, and 5% β-2-mercaptoethanol in 0.08 M Tris-buffer, pH 6.8, were loaded on SDS-PAGE slab gels.

SDS-PAGE. SDS-PAGE was performed in slabs as described by Laemmli (16). The stacking and separating gel combinations used were 5 and 7.5%. To calibrate the gels for molecular weight estimation, we used proteins from a kit purchased from Pharmacia Fine Chemicals,

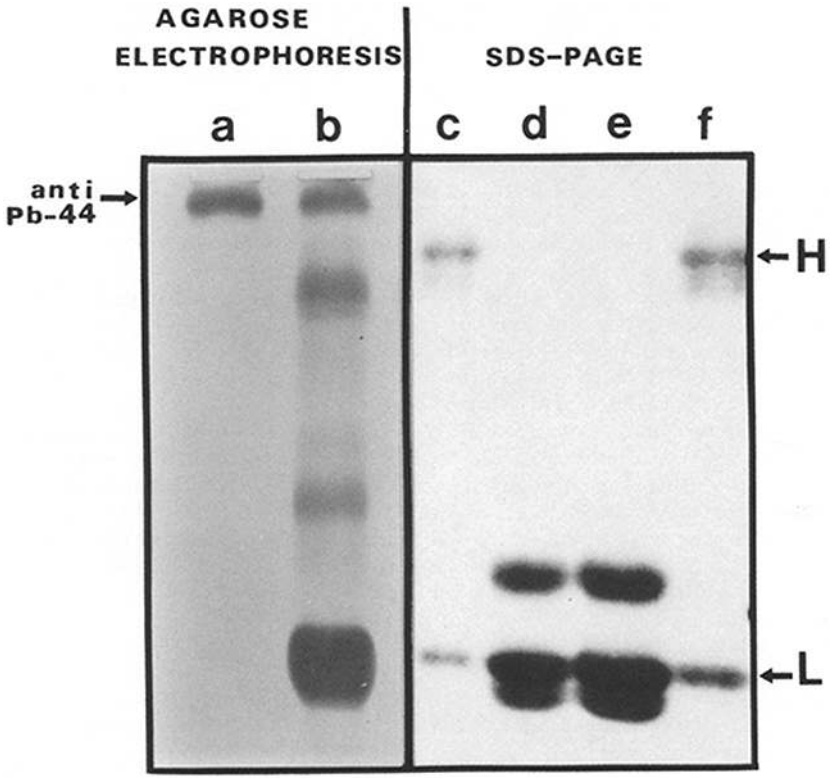

FIG. 1. Characterization of purified monoclonal antibodies to Pb44 and its papain digest. On the left are results of high-voltage electrophoresis in agarose of ascitic fluid from a mouse that bore the hybridoma tumor (track b), and the purified monoclonal antibodies to Pb44 (track a). On the right, SDS-PAGE under reducing conditions of the native monoclonal antibodies (tracks c and f), and of the papain digest (tracks d and e). Notice that the tracks that contained the digest were overloaded to verify the absence of intact heavy (H) chains. These tracks show heavy-chain fragments in addition to the expected light (L) chains.

Div. of Pharmacia, Inc. (range of molecular weights from 14,400 to 94,000). After electrophoresis, the gels were stained with 0.04% Coomassie blue in 10% methanol and 7% acetic acid. Destaining was carried out with a 30% methanol, 7% acetic acid solution. Photographs of the stained gels were obtained after drying. Radioautography was carried out by exposing the dried gels to an X-omat R film (XR5) and an image-intensifying screen (Cronex; DuPont Instruments, Wilmington, Del.) at −70°C.

Agarose Electrophoresis. Agarose electrophoresis was performed in 1-mm thick agarose gel (1.1%) in veronal buffer, pH 8.6, as described by Johansson (17).

Results

Isolation and Characterization of Monoclonal Antibodies to Pb44. There was significant growth of cell colonies in 575 out of 859 wells seeded after fusion of the plasmacytoma cell line with spleen cells from mice immunized with *P. berghei* sporozoites. Five wells yielded supernates that contained antibodies to sporozoites as detected by indirect immunofluorescence. Only one (3D11) however, was successfully expanded and cloned by the limiting-dilution method. We used thymocytes from adult BALB/c mice (10^6 cells per well) as feeder cells during cloning. Out of six clones tested, five were positive by immunofluorescence. These five supernates were used to immunoprecipitate extracts of ^{125}I-surface-labeled sporozoites of *P. berghei* by the Kessler (14) procedure. As shown in Fig. 2, all reacted against Pb44.

Monoclonal antibodies to Pb44 were isolated from the ascitic fluid of mice that bore the hybridoma tumor and analyzed by high-voltage agarose-gel electrophoresis and by double diffusion using specific antisera to mouse immunoglobulin heavy and light chains. We characterized the antibodies as β-globulins containing $\gamma 1$, κ chains.

Protective Effects of Antibodies to Pb44. The first indication that the monoclonal antibodies to Pb44 had a protective effect in vivo came from experiments in which mice that bore the hybridoma, but not the parent plasmacytoma, were resistant to malaria, i.e., they failed to develop infection upon challenge with 2.5×10^4 sporozoites (Table I).

To establish that indeed the antibodies secreted by the tumor were preventing infection, several groups of A/J mice were injected intravenously with decreasing amounts of purified antibody, and they were challenged with sporozoites 30 min later. In mice challenged with 10^3 sporozoites, almost complete protection was obtained with the smallest doses of antibody used (10–50 μg). In animals receiving 10^4 sporozoites, only the highest dose of antibody (300 μg) was fully protective. In all groups that received antibody and in which parasitemia was observed, the prepatent period was prolonged in relation to controls (Table II).

To clarify the mechanism of the antibody-mediated protection, we compared the effects on sporozoites of Fab fragments and intact antibodies. Because the fragments and native molecules have very different clearance rates (18), we used the sporozoite-neutralizing test for this comparison. *P. berghei* sporozoites were incubated with different concentrations of the reagents for 45 min at room temperature and injected into mice. The results (Table III) showed that Fab and the intact antibody are almost equally effective in their neutralizing activity.

That the effect of Fab fragments on sporozoites was specific is shown by the lack of neutralizing activity of the same preparation on sporozoites of *P. yoelii nigeriensis,* a different species of rodent malaria. Also, *P. berghei* sporozoites incubated in Fab (1 mg/ml) prepared from nonspecific rabbit gamma globulin remained fully infective.

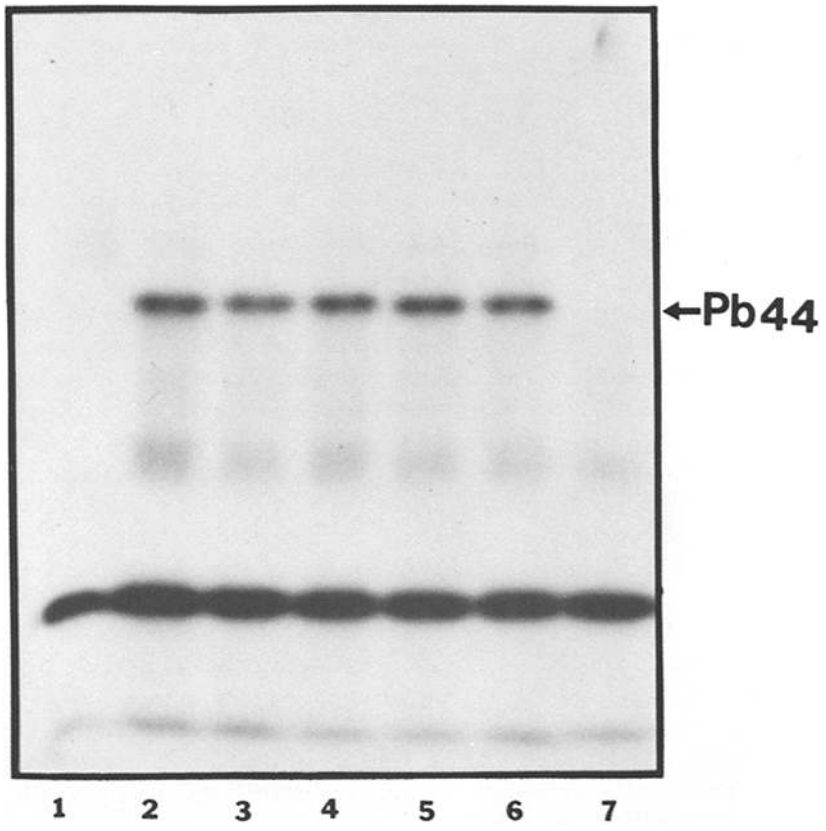

FIG. 2. SDS-PAGE of immunoprecipitates resulting from the reaction of labeled sporozoites with supernates from cultured clones of hybridoma cells. The surface membrane of sporozoites was radiolabeled with ^{125}I and solubilized in detergent. Portions of the labeled extracts were incubated with culture fluids from wells that contained clones of hybridoma cells. The immune complexes were bound to Staph A, eluted with sample buffer that contained denaturing and reducing agents, and loaded in the gel. Shown are the results of the radioautography of the dried gel. Tracks 2–6 originate from immunoprecipitation with supernates from five different clones. Tracks 1 and 7 are controls obtained after immunoprecipitation of the extracts with normal mouse serum or with MOPC-21 myeloma protein (IgG1, κ), respectively. Note that a single specific band was immunoprecipitated by the supernates derived from the clones (Pb44). The apparent molecular weight (42,000–44,000) was determined by comparison of its electrophoretic mobility with that of standards of known molecular weights.

The Mechanism of the CSP Reaction. Next we investigated the role of Pb44 in the CSP reaction. As shown in Table IV, monoclonal antibodies at concentrations between 10 and 20 μg/ml induce the tail-like precipitate at the posterior end of the viable sporozoite. The reaction is specific: no morphological changes were observed upon incubation of *P. yoelii yoelii, P. yoelii nigeriensis* or *P. chabaudi* sporozoites with the antibodies. Fab fragments of antibodies to Pb44 alone were totally ineffective in inducing CSP reactions, even at high concentrations (1,500 μg/ml). However, strong CSP reactions occurred if, after incubation with low concentrations of Fab (1–2 μg/ml), the sporozoites were treated with rabbit antibodies to mouse κ-chains. The binding of Fab to the surface membrane of the parasite could also be demonstrated by indirect immunofluorescence.

TABLE I

Mice That Bear the Hybridoma Tumor 3D11 Are Resistant to Infection with P. berghei Sporozoites

Experiment	Tumor cells injected*	Result of challenge of mice with sporozoites; number infected/number challenged
I	Hybridoma 3D11 anti-Pb44	0/10
	Plasmacytoma P3U1	3/4
	None	5/5
II	Hybridoma 3D11 anti-Pb44	0/10
	Plasmacytoma P3U1	4/5
	None	4/5

* Solid tumors were induced in BALB/c mice by subcutaneous injection of 5×10^6 cells. 2 wk later, when the tumors were ~1 cm in diameter, the mice were challenged with 5×10^3 or 25×10^3 sporozoites in experiments I and II, respectively.

TABLE II

*Resistance to Infection with P. berghei Sporozoites of Mice Injected with Purified Monoclonal Antibodies to Pb44**

Dose of antibodies	Challenged with 10^3 sporozoites		Challenged with 10^4 sporozoites	
	Number infected/Number challenged	Prepatent period	Number infected/Number challenged	Prepatent period
μg/mouse		*d ± SE*		*d ± SE*
300	0/5	—	0/5	—
100	0/5	—	4/5	5.7 ± 0.2
50	1/5	7.0	5/5	5.2 ± 0.2
25	1/5	6.0	5/5	5.2 ± 0.2
10	0/5	—	5/5	4.6 ± 0.4
None	5/5	5.2 ± 0.4	5/5	4.0 ± 0

* Purified antibody was administered intravenously to A/J mice 30 min before challenge with either 10^3 or 10^4 sporozoites.

Discussion

Our findings demonstrate that monospecific antibodies (IgG1, κ) directed against a surface component (Pb44) of *P. berghei* sporozoites confer complete protection to mice against an otherwise lethal inoculum of parasites. The degree of protection is a function of the number of parasites used in the challenge and of the antibody concentration. Mice that bore the hybridoma tumor, whose serum concentration of antibodies to Pb44 was ~2 mg/ml, were completely protected against infection with 2.5×10^4 sporozoites (Table I). Passive transfer of relatively small amounts of antibody (10 μg) abolished or profoundly diminished the infectivity of 10^3 parasites, but much greater amounts of antibody (300 μg) were required for complete protection against challenge with 10^4 sporozoites. The effects of antibody were also clearly reflected in

TABLE III

*Neutralization of P. berghei by Treatment In Vitro with Monoclonal Antibodies to Pb44 or Their Fab Fragments**

Parasite (sporozoites)	Treatment with anti-Pb44		Result of challenge of mice; number infected/ number challenged
	IgG	Fab	
	μg/ml		
P. berghei	1,200	—	0/10
	240	—	3/15
	48	—	5/10
	24	—	3/5
	12	—	4/5
P. berghei	—	1,200	0/15
	—	240	0/15
	—	48	0/9
	—	24	5/5
	—	12	5/5
P. berghei	Control‡		19/20
P. yoelii nigeriensis	—	1,200	5/5
P. yoelii nigeriensis	Control‡		5/5

* 10^5 sporozoites were incubated for 45 min at room temperature with native monoclonal antibodies to Pb44 or Fab fragments in 0.5 ml of PBS that contained 10% of normal mouse serum. After incubation, 10^4 sporozoites were injected intravenously into each A/J mouse. The table summarizes the results of three separate experiments.

‡ No antibody.

TABLE IV

P. berghei CSP Reactions Induced by Monoclonal Antibodies to Pb44 and by Their Fab Fragments

Reagent	CSP titer*	
	Direct	Indirect
Ig anti-Pb44	1/128	—
Fab anti-Pb44	Negative	1/1,024
Medium (control)‡	Negative	Negative

* Maximal dilution of antibodies or Fab fragments giving a positive CSP reaction as described in Materials and Methods. The initial concentrations of native antibody and Fab fragments were identical (1.5 mg/ml). After incubation with the Fab dilutions or with medium (control), the sporozoites were washed once with Medium 199 and reincubated for an additional 30 minutes with 50 μl of a 1/10 dilution of a rabbit antiserum to mouse κ-chains (indirect CSP reaction).

‡ Controls were incubated with Medium 199 that contained 10% normal mouse serum.

the significant prolongation of the prepatent periods in all animals that became infected (Table II). Taking into consideration that a small number of parasites is usually injected by mosquito bite (19), our findings are encouraging for the prospect of developing a malaria vaccine using purified sporozoite membrane antigens.

It should be pointed out, however, that mice actively immunized by the injection of radiation-attenuated sporozoites are fully protected against challenge with up to 10^5 sporozoites, and may have relatively low antibody titers, as determined by CSP reactions (20). The reason for the greater effectiveness of the vaccine as compared to passive administration of antibodies to Pb44 is not clear. It is possible that protection in vaccinated mice is mediated by antigens other than Pb44. Alternatively, antibodies to Pb44 of other classes and/or of other specificities with higher binding affinity may be present in the vaccinated mice, and these may be more efficient in protection. (The present hybridoma is of the IgG1 subclass, which presumably does not activate the classical complement pathway [21].) Also, in actively immunized animals, the protective role of antibodies is probably enhanced by cellular mechanisms. Some of these questions may hopefully be answered by developing and characterizing the properties of additional monoclonal antibodies to *P. berghei*, and by studying the protective effects of vaccination with purified Pb44.

The neutralizing activity of antibodies to Pb44 may be mediated through the primary interaction, that is, an effect on the parasite itself and/or could result from secondary effects such as complement fixation, phagocytosis, killer-cell activity, etc. The finding that Fab fragments are as effective in mediating protection as the native molecule (Table III) strongly suggests that the antibodies interfere with a parasite function necessary for its infectivity, such as, for example, the ability to penetrate into the target cell or to proliferate in the hepatocytes after penetration.

Our observations also shed light on the nature of the CSP reaction. This reaction, which consists of the formation of a thread-like precipitate at the posterior end of the sporozoites (4, 7) occurred after direct interaction of the parasites with the intact monoclonal antibody to Pb44. The monovalent (Fab) antibody fragments alone, even at very high concentrations, were ineffective, but the reaction could be readily produced by treating sporozoites with small concentrations of Fab followed by incubation with rabbit antibodies to mouse κ-chains. We conclude that the CSP reaction can result from the cross-linking of Pb44 and that its characteristics are those of a capping reaction followed by shedding of the immune complexes (22).

The significance of the CSP reaction is not clear. It could represent a mechanism of escape of the parasite from the host immune response. The efficacy of such an escape mechanism is questionable. The antigen-antibody complexes formed on the surface of the sporozoites remain fully accessible to effector mechanisms which would tend to eliminate the parasites. Moreover, our data show that the CSP reaction does not protect the parasite, but rather the host, because at high concentrations of antibody, when the CSP reaction is strongest, sporozoite challenge fails to induce infection.

An intriguing possibility is that the CSP reaction mimics the events that occur when the parasites encounter the target cell. In other words, the antibodies to Pb44 could be artificially triggering a reaction, which under normal circumstances would take place when Pb44 molecules encounter an appropriate divalent receptor on the membrane of the target cells, presumably hepatocytes. Perhaps by binding to Pb44, antibodies would render these molecules inaccessible to the putative hepatocyte receptor and thus interfere with sporozoite infectivity.

We are currently using the monoclonal antibodies to purify Pb44 to determine whether the isolated protein is immunogenic and can be used as a vaccine. If

successful, this would encourage similar attempts to characterize and isolate the protective antigen(s) of sporozoites of human malaria.

Summary

Monoclonal antibodies (IgG1, κ) directed against a surface component of *Plasmodium berghei* sporozoites (Pb44) confer complete protection to mice against a lethal inoculum of parasites. The degree of protection is a function of the number of parasites used in the challenge and of the antibody concentration in serum. Passive transfer of 10 μg of antibody per mouse abolished or profoundly diminished the infectivity of 10^3 sporozoites, but much higher amounts of antibody were required for complete protection against challenge with 10^4 parasites.

Fab fragments of the monoclonal antibodies were as effective as the intact antibodies in mediating protection as determined by the neutralizing assay. This observation suggests that the antibodies interfere with a parasite function necessary for its infectivity, such as, for example, the ability to penetrate into the target cell or to multiply in the hepatocytes.

When sporozoites are incubated with the intact monoclonal antibodies at 37°C, a long filament appears at its posterior end (circumsporzoite precipitation [CSP] reaction). Fab fragments are ineffective at high concentrations. However, if after treatment with Fab, the sporozoites are incubated with rabbit antibodies to mouse κ-chains, a strong CSP reaction is observed. We conclude that the CSP reaction can result from the cross-linking of Pb44 and that it has the characteristics of a capping reaction followed by the shedding of the immune complexes.

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