INFECTION AND IMMUNITY, Apr. 1986, p. 314–318
0019-9567/86/040314-05$02.00/0

Vol. 52, No. 1

NOTES

Induction of Protective Immunity to Monoclonal-Antibody-Defined *Plasmodium falciparum* Antigens Requires Strong Adjuvant in *Aotus* Monkeys

WASIM A. SIDDIQUI,* LESLIE Q. TAM, SIU-CHOW KAN, KENTON J. KRAMER, STEVEN E. CASE, KEVIN L. PALMER, KAREN M. YAMAGA, AND GEORGE S. N. HUI

Department of Tropical Medicine and Medical Microbiology, John A. Burns School of Medicine, Leahi Hospital, University of Hawaii, Honolulu, Hawaii 96822

Received 16 September 1985/Accepted 16 December 1985

Monoclonal antibodies to the major *Plasmodium falciparum* merozoite surface coat and rhoptry antigens were produced. A combination of the affinity-purified polypeptides with Freund complete adjuvant which was given three times completely protected an *Aotus lemurinus azure* (karyotype VI) monkey against homologous challenge; however, immunization with the same polypeptides with a muramyl dipeptide derivative [MDP-Lys(L18)] did not protect a second *Aotus* monkey, even though comparable high antibody titers were induced.

Vaccination with schizonts or merozoites of the human malaria parasite *Plasmodium falciparum* has been shown to protect *Aotus* (owl) monkeys against lethal challenge infection (17, 26). The identification of defined, protective polypeptides on which a recombinant or synthetic peptide vaccine may be based is currently being investigated in numerous laboratories. A processed, merozoite surface coat precursor protein, having molecular weight 185,000 to 200,000 (185K to 200K protein) is the most well characterized of the *P. falciparum* antigens (5–9, 11–13, 16, 22) and has been shown to be partially protective in *Saimiri* (squirrel) monkeys (6, 20). Recently, antigens associated with the merozoite rhoptry, an organelle involved in merozoite invasion, have gained attention because of both the strong protection induced in squirrel monkeys by a 41K rhoptry polypeptide (19) and the report that a ring-infected erythrocyte surface antigen originates in the rhoptry micronemes (1). Moreover, polyclonal antibodies to ring-infected erythrocyte surface antigens (31) and monoclonal antibodies to rhoptry antigens (21) are inhibitory in vitro. In each of the vaccination experiments, polypeptides were given in Freund complete adjuvant (FCA) followed by two boosters in Freund incomplete adjuvant. For clinical use, FCA is not acceptable, and it is not known whether the synthetic adjuvants now available will be effective with these defined polypeptides. To date, these polypeptides have not been shown to induce protection in owl monkeys, in which lethal infection with some *P. falciparum* isolates has not required lengthy adaption, in contrast to squirrel monkeys. We report here an observation in which a combination of the major *P. falciparum* merozoite surface coat and rhoptry antigens, isolated by monoclonal antibodies, protected an *Aotus* karyotype VI monkey when given in FCA, but not when given with a muramyl dipeptide (MDP) derivative [MDP-Lys(L18)]. The observation provides evidence that strong adjuvants may be required for a successful human malaria vaccine consisting of defined antigens or epitopes.

Hybridomas were produced by fusion of spleen cells from BALB/c mice immunized with schizont-enriched *P. falciparum* with P3x63Ag8.653 myeloma cells. Hybridomas were screened by solid-phase radioimmunoassay (14); positives were double cloned, and ascites were then produced. Antibody 5.2 stained schizonts and free merozoites by indirect immunofluorescence on acetone-fixed parasites. The pattern was even, linear staining of the parasite surface (Fig. 1a). Antibody 219.5 produced a dotted immunofluorescence pattern on schizonts and free merozoites. The dots appeared as pairs, and staining occurred on a limited region of the parasite approximating the apical rhoptry organelles (Fig. 1b). Antibodies 5.2 (IgG2b) and 219.5 (IgG1) were purified on Protein-A Sepharose and DEAE-Sephacel (Pharmacia Fine Chemicals) respectively, and high-capacity immunoabsorbents were prepared (24). Similarly, immune immunoglobulin G (IgG) from previously vaccinated and protected *Aotus* monkeys was covalently bonded to Protein-A Sepharose.

Serum samples from immune *Aotus* monkeys immunoprecipitate at least 21 metabolically labeled *P. falciparum* asexual blood stage antigens (13). Antibodies 5.2 and 219.5 were found to coidentify with immune *Aotus* IgG five prominent and other minor metabolically labeled polypeptides (Fig. 2). Antibody 5.2 immunoabsorbent bound a prominent 185K polypeptide and its minor 152K, 121K, and 83K proteolytic processing fragments (Fig. 2, lane 1). The 83K polypeptide is one of three processing fragments on the merozoite surface (5, 9). Antibody 219.5 bound 143K and 132K polypeptides (Fig. 2, lane 3). These polypeptides were also immunoprecipitated by antibody 61.3, obtained from Wellcome Biotechnology (data not shown). The *P. falciparum* Welcome Lagos polypeptides (155K and 140K) isolated are bound by antibody 61.3 and are noncovalently associated (10). The 140K polypeptide has been localized by immune electron microscopy in the rhoptry (10). Similar

* Corresponding author.

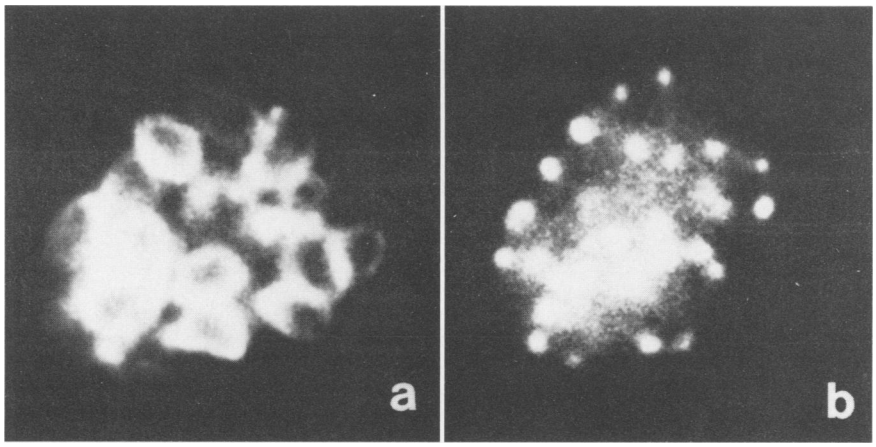

FIG. 1. Indirect immunofluorescence assay of acetone-fixed *P. falciparum* schizonts stained with antibody 5.2 to the parasite surface (a) and antibody 219.5 to the rhoptry organelles (b).

antigens have been identified by other monoclonal antibodies (2).

The *P. falciparum* Uganda Palo Alto (FUP)-K^+ (monkey passaged) isolate was grown in a semiautomated in vitro culture system (18). The K^+ parasites were not kept in culture for more than 8 weeks (32). Approximately 8×10^{11} infected erythrocytes, (~33% each of rings, trophozoites, and schizonts) were lysed with 0.013% saponin, and centrifuged parasites were extracted with 7 volumes of 1% Nonidet P-40–10 mM iodoacetamide–5 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid]–5 mM EDTA–1 mM phenylmethylsulfonyl fluoride in borate-buffered saline (pH 8.0). About 500 mg of crude protein (Bio-Rad protein assay; Bio-Rad Laboratories) was obtained. The extract was divided into two aliquots, and each was supplemented with [^{35}S]methionine-labeled polypeptides. One aliquot was passed serially through 3 ml of antibody 5.2 and then 3 ml of antibody 219.5 immunoabsorbents. The other aliquot was passed through the immune *Aotus* IgG immunoabsorbent. The columns were washed with 0.5 M NaCl-borate buffer and eluted with 50 mM diethylamine (pH 11.5) containing 5 mM iodoacetamide, 1 mM EDTA, 1 mM EGTA, and 0.1% Nonidet P-40. Collected fractions were neutralized with 1 M Tris hydrochloride (pH 8.0), and the peak radioactive tubes were pooled and dialyzed against borate-saline (pH 8.0). Immune *Aotus* IgG, antibody 5.2, and antibody 219.5 immunoabsorbents isolated 1.8, 0.62, and 0.23 mg of protein, respectively.

In previous vaccination experiments (26–29), we used *Aotus lemurinus griseimembra* (karyotype II and III) monkeys, in which the *P. falciparum* FUP-K^+ isolate causes a 70 to 80% parasitemia and in which the infection is lethal, if not treated with drugs. Because *Aotus* monkeys of this karyotype are scarce, a small experiment was performed in *Aotus lemurinus azure* (karyotype VI), in which the FUP-K^+ isolate consistently produces a 2 to 4% parasitemia. The monkeys were given 80 μg of antibody 5.2-isolated polypeptides in combination with 30 μg of antibody 219.5-isolated polypeptides (animal A381), 240 μg of the total parasite polypeptides isolated by immune *Aotus* IgG (animal A391), or 1.4 mg of unpurified parasite material (animal A390). The antigens were given three times in 1 ml of FCA emulsion (GIBCO) at 21-day intervals at multiple sites intramuscularly and subcutaneously. The amount of mycobacterium was successively halved on secondary and tertiary immunizations. A fourth *Aotus* monkey (A386) received the same amount of monoclonal-antibody-isolated polypeptides as did A381 (at a 3× concentration) but with

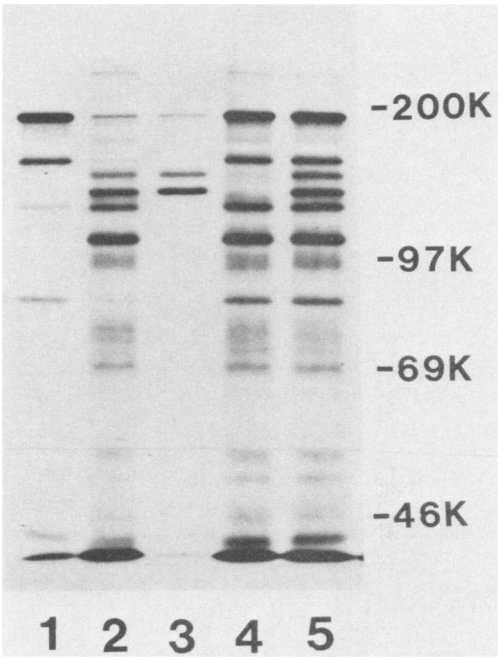

FIG. 2. Coidentification of [^{3}H]isoleucine-labeled *P. falciparum* polypeptides by monoclonal antibody 5.2 to the parasite surface and monoclonal antibody 219.5 to the parasite rhoptry organelles with polypeptides bound by immune *Aotus* IgG. The metabolically labeled polypeptides were bound to antibody 5.2 (lane 1) or antibody 219.5 (lane 3) immunoabsorbents, eluted, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 6.25% gel. The unbound polypeptides passing through the monoclonal antibody 5.2 and 219.5 immunoabsorbents were then adsorbed to immune *Aotus* IgG immunoabsorbent, eluted, and analyzed (lanes 2 and 4, respectively). Total polypeptides bound by immune *Aotus* IgG are shown in lane 5. The molecular weight markers are myosin (200K), phosphorylase *b* (97K), bovine albumin (69K), and ovalbumin (46K) (New England Nuclear Corp.).

TABLE 1. Immune response and parasitemia in karyotype VI *Aotus* monkeys immunized with *P. falciparum* merozoite surface and rhoptry polypeptides and challenged with FUP-K$^+$

Animal no.	Antigens	Adjuvant	Antibody titers[a]		No. of parasites/ 10,000 erythrocytes
			IIFA	RIA	
A385	None	None	16	250	220[b]
A386	Surface + rhoptry	MDP-Lys(L18)	2,056	31,250	380[c]
A381	Surface + rhoptry	FCA	4,096	31,250	0[d]
A391	Total IgG isolated	FCA	8,192	31,250	0[d]
A390	Whole cell	FCA	8,192	>31,250	0[d]

[a] Reciprocal dilution of the highest 14-day tertiary serum yielding a positive result. IIFA, Indirect immunofluorescence assay; RIA, radio immunoassay.
[b] Monkey A385 became patent on day 4, developed a peak parasitemia of 2.2% on day 13, and cleared the infection on day 17.
[c] Monkey A386 became patent on day 5, developed a peak parasitemia of 3.8% on day 9, and cleared the infection on day 17.
[d] No parasites were detected up to 60 days after challenge.

200 μg of an MDP derivative [MDP-Lys(L18)] in 1.5 ml of Intralipid (Cutter Medical) instead of FCA. This adjuvant has been shown to be effective with some bacterial and fungal infections (15). A fifth *Aotus* monkey (A385) was a nonimmunized control. Serum samples were obtained 14 days after each immunization. Tertiary serum antibody titers are shown in Table 1, and the immunoprecipitated [^{35}S]methionine-labeled polypeptides are shown in Fig. 3.

All *Aotus* monkeys were challenged 3 weeks after the last immunization with 10^6 FUP-K$^+$ parasites twice passaged in *Aotus* monkeys. The unimmunized control (A385) and the monkey given polypeptides with the MDP derivative (A386) developed a patent infection with low-level parasitemia, characteristic of nonimmune *Aotus* monkeys of this karyotype; however, all monkeys immunized with polypeptides in FCA did not reveal parasites in thick blood smears when monitored daily over a 2-month period (Table 1). In earlier studies, we infected six other *Aotus* karyotype VI monkeys with the FUP-K$^+$ isolate in karyotype adaptation experiments. In the course of these experiments, a breakthrough in parasitemia to the 2 to 4% level was always observed in each of the animals. Therefore, we are confident that the animals immunized with the antigens in FCA in this small experiment were completely protected.

Some information is available on the efficacy of synthetic adjuvants in replacing FCA for use with malaria vaccines. Unmodified MDP was either unable (23, 29) to replace FCA in vaccination experiments involving merozoite-enriched *P. falciparum* preparations or had only limited success (30). However, replacement of the primary hydroxyl group at the C-6 position of MDP with a stearoyl group resulted in significant protection (28). In addition, all *Aotus* monkeys vaccinated with schizont-enriched *P. falciparum* containing CP-20,961 synthetic adjuvant survived challenge but had moderate parasitemias (27). Thus, synthetic adjuvants have been used successfully in the past with crude parasite

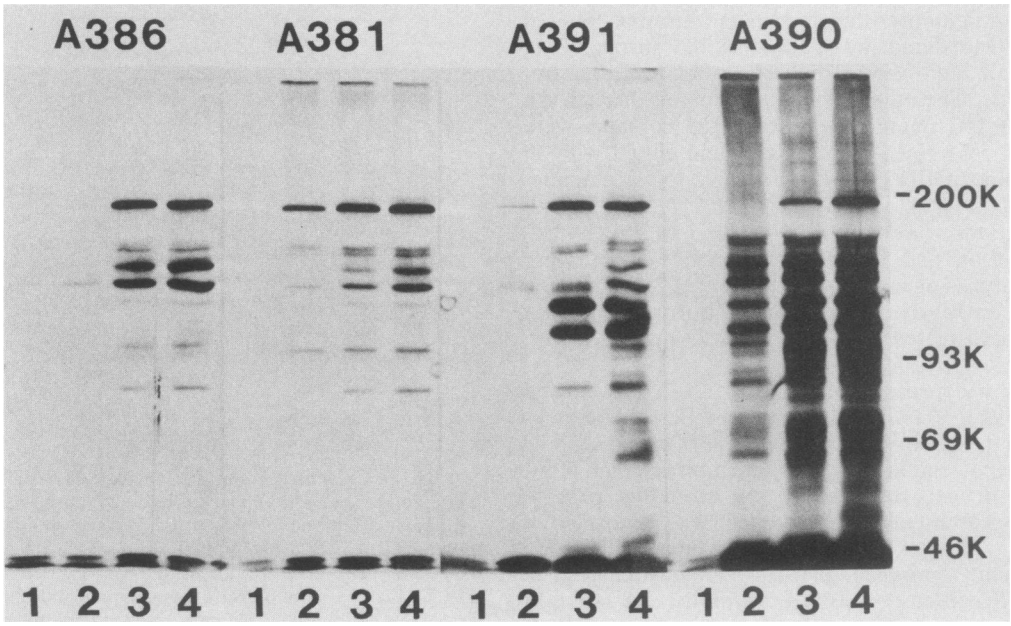

FIG. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of [^{35}S]methionine-labeled *P. falciparum* polypeptides immunoprecipitated by tertiary sera from *Aotus* monkeys vaccinated with *P. falciparum* polypeptides. The polypeptides were complexed with 7.5 μl of preimmune (lane 1), primary (lane 2), secondary (lane 3), or tertiary (lane 4) serum from *Aotus* monkeys immunized with a combination of surface and rhoptry polypeptides in an MDP derivative [MDP-Lys(L18)] (animal A386) or FCA (animal A381), the set of polypeptides affinity-purified by immune *Aotus* IgG in FCA (animal A391), or unpurified schizont-enriched parasite material in FCA (animal A390). Complexes were collected with Formalin-treated *Staphylococcus* A, and polypeptides were electrophoresed in 6.25% gel. The molecular weight standards are the same as in Fig. 2, except that phosphorylase *b* is 93K (Amersham Corp.).

material but are not yet approved for human use. The MDP-Lys(L18) used in this experiment was selected because of its low pyrogenicity and arthritogenicity; however, it also lacked sufficient adjuvanticity.

It is known that immunity to malaria in holoendemic areas with high transmission is only partial and that semi-immune adults can experience repeated malaria infections in the presence of circulating antibody. This coexistence of blood parasites with serum antibody is termed premunition. The *Aotus* monkeys vaccinated with merozoite surface coat and rhoptry polypeptides in synthetic adjuvant produced high levels of antibody; however, the immune response had no effect on parasitemia. It is possible that protective antibody in nature is directed at weakly immunogenic or antigenically diverse epitopes. Levels adequate for complete protection might be induced only after repeated infections in nature or with strong adjuvants under experimental conditions.

The ability of FCA to induce an antibody response of greater heterogeneity than that induced by unmodified MDP has recently been reported with a synthetic influenza peptide-tetanus toxoid conjugate (25). Mice immunized with the conjugate in FCA or MDP had equivalent antibody titers against the synthetic peptide. However, only mice immunized with the conjugate in FCA made antibody to an epitope found on the native virus. A similar situation may exist with the *P. falciparum* merozoite surface coat and rhoptry polypeptides. Although the antibody titers were similar in *Aotus* monkeys immunized with the polypeptides in FCA and in MDP-Lys(L18), the epitope specificity of these sera is not known. It is possible that sera from the *Aotus* monkeys immunized with the polypeptides in FCA contained higher levels of antibody to weakly immunogenic, but critically protective epitopes. Such epitopes might be formed by repeat sequences on these molecules. With the S antigens of *P. falciparum*, the repeat sequences vary among isolates, are weakly immunogenic, and have been postulated to enable the parasite to evade the host's immune system (3). Polymorphism has been demonstrated with the merozoite surface coat protein (16), although the repeat sequence is not extensive (11). It is not known whether the rhoptry polypeptides bear repeat sequences.

It must be cautioned that the result described here was obtained with a single animal. Nevertheless, this is the first report of protection by defined *P. falciparum* polypeptides in the *Aotus* monkey. Heretofore, protection has been demonstrated in *Saimiri* monkeys (4, 6, 19, 20). The strong dependence of protection on FCA provides evidence that a successful malaria vaccine may be dependent not only on the identification and cloning of protective antigens and epitopes, but also on the development of effective synthetic adjuvants suitable for use in humans.

We thank S. Kotani, Osaka University Dental School, for the MDP-Lys(L18) and R. R. Freeman, Wellcome Biotechnology, Ltd., for the monoclonal antibody 61.3. The technical assistance of Ann Kato and JoAnn Olejarczyck is gratefully appreciated.

This investigation was supported, in part, by the U.S. Agency for International Development through contract DPE 0453-C-00-1026.

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