Partial Protection against Plasmodium vivax Blood-Stage Infection in Saimiri Monkeys by Immunization with a Recombinant C-Terminal Fragment of Merozoite Surface Protein 1 in Block Copolymer Adjuvant

CHUNFU YANG,1 WILLIAM E. COLLINS,1 JOANN S. SULLIVAN,1 DAVID C. KASLOW,2 LIHUA XIAO,1 AND ALTAF A. LAL1*

Division of Parasitic Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Public Health Service, U.S. Department of Health and Human Services, Atlanta, Georgia 30341,1 and Laboratory of Malaria Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 208922

Received 26 May 1998/Returned for modification 24 July 1998/Accepted 1 October 1998

Merozoite surface protein 1 is a candidate for blood-stage vaccines against malaria parasites. We report here an immunization study of Saimiri monkeys with a yeast-expressed recombinant protein containing the C terminus of Plasmodium vivax merozoite surface protein 1 and two T-helper epitopes of tetanus toxin (ypP30Pv20019), formulated in aluminum hydroxide (alum) and block copolymer P1005. Monkeys immunized three times with ypP30Pv20019 in block copolymer P1005 had significantly higher prechallenge titers of immunoglobulin G (IgG) antibodies against the immunogen and asexual blood-stage parasites than those immunized with ypP30Pv20019 in alum, antigen alone, or phosphate-buffered saline (PBS) (P < 0.05). Their peripheral blood mononuclear cell proliferative responses to immunogen stimulation 4 weeks after the second immunization were also significantly higher than those from the PBS control group (P < 0.05). Upon challenge with 100,000 asexual blood-stage parasites 5 weeks after the last immunization, monkeys immunized with ypP30Pv20019 in block copolymer P1005 had prepatent periods longer than those for the control alone group (P > 0.05). Three of the five animals in this group also had low parasitemia (peak parasitemia, ≤20 parasites/μl of blood). Partially protected monkeys had significantly higher levels of prechallenge antibodies against the immunogen than those unprotected (P < 0.05). There was also a positive correlation between the prepatent period and titers of IgG antibodies against the immunogen and asexual blood-stage parasites and a negative correlation between accumulated parasitemia and titers of IgG antibodies against the immunogen (P < 0.05). These results indicate that when combined with block copolymer and potent T-helper epitopes, the yeast-expressed P.P30Pv20019 recombinant protein may offer some protection against malaria.

Plasmodium vivax is one of the most widely distributed human malaria parasites, prevalent in South America, Asia, and Oceania (27). With the appearance of resistance to current antimalarial drugs (9), an effective vaccine against the parasite is urgently needed. Several antigens expressed at different stages of the parasitic life cycle have been characterized and found to have the potential for use in a subunit vaccine against P. vivax (2, 15, 32, 37). One of these antigens, merozoite surface protein 1 (MSP-1), is considered a leading candidate for asexual blood-stage vaccines against malaria parasites (27, 29, 30). One way to influence the host immune responses to C-terminal fragments of MSP-1 is to formulate it in adjuvants (22, 40). In vitro and in vivo studies with P. falciparum (3, 7, 8, 10, 20, 26, 33) and P. yoelii (6, 11, 28, 30, 34, 42) have shown that immunoglobulin G (IgG) antibodies or monoclonal antibodies directed against the C-terminal 19-kDa fragment can inhibit the invasion of parasites into erythrocytes or protect mice or monkeys against live parasite challenges. Field studies have also shown that production of IgG antibodies against the 19-kDa fragment of P. falciparum correlates with the development of clinical immunity against falciparum malaria (17, 35, 36). Taken together, these findings suggest that the C-terminal 19-kDa fragment of MSP-1 is a vaccine candidate antigen against asexual blood-stages of malaria parasites.

Both humoral and cellular immune responses are necessary for an effective malaria vaccine against blood-stage parasites (29, 40). One way to influence the host immune responses to an antigen is by the use of adjuvants (1, 19, 23, 25, 45). Immunization studies with C-terminal fragments of P. falciparum MSP-1 in primate malaria models showed that no protection was induced when an Escherichia coli-expressed 19-kDa antigen was formulated in aluminum hydroxide (alum) and liposomes (5). However, protection was achieved when a baculovirus-expressed 42-kDa or yeast-expressed 19-kDa antigen
mixed with the Freund's complete adjuvant (7, 26). A recent report has also shown that rhesus monkeys immunized with baculovirus-expressed 42- or 19-kDa antigen of *P. cynomolgi* MSP-1 in Freund's complete or incomplete adjuvants were protected (32). Thus, adjuvants affect the efficacy of recombinant MSP-1 vaccines. Unfortunately, Freund's adjuvant is too toxic for human use, and alum is currently the only approved human-usable adjuvant.

One adjuvant currently under development for use in humans is the nonionic block copolymer, which is a simple linear chain of the hydrophobic polyoxyethylene flanked by two chains of the hydrophilic polyoxyethylene (22). Antibodies bind to the hydrophobic copolymers by hydrophobic and hydrogen bond interactions. Our studies with different formulations of nonionic block copolymers P1004 and P1005 with malaria antigens have shown that they can modulate both humoral and cellular immune responses, resulting in different outcomes of challenge infections (21, 41, 46, 47).

In contrast to the intensive studies done on *P. falciparum* and *P. yoelii* MSP-1, little is known about *P. vivax* MSP-1. Our previous study of mice with a yeast-expressed 19-kDa antigen of *P. vivax* MSP-1 formulated in nonionic block copolymer P1005 showed that this formulation was highly immunogenic. Mice produced high antibody and proliferative responses comparable to those induced by using Freund's complete adjuvant (46). In this study, we further evaluated the immunogenicity of this yeast-expressed *P. vivax* MSP-1 19-kDa fragment in *Saimiri* monkeys and assessed the protective effect of immunizations with this recombinant protein in the human-useable adjuvant alum and a potentially usable adjuvant block copolymer.

**MATERIALS AND METHODS**

**Antigen.** The antigen was a yeast-expressed recombinant protein, yP2P30Pv20019, consisting of the C terminus (amino acids Asn 1622 to Ser 1729) of the nonionic block copolymer P1005, however, developed skin sores at the injection sites and was stopped by the addition of 50% of tetramethylbenzidine-peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, Md.). The reaction was stopped by the addition of 50 μl of 1 M H3PO4 and read at 450nm with a microplate reader. Titers were based on the highest dilution of the sample that generated an optical density greater than the mean of preimmunization sera plus 2 standard deviations (cutoff optical density = 0.805).

**Asexual blood-stage parasite challenge.** Antibodies against P. vivax 19-kDa blood-stage parasites 1 week after the third immunization, on the day of the asexual blood-stage parasite challenge (week 0) and 2 and 4 weeks after the asexual blood-stage parasite challenge were determined by IFA as described by Sulzer et al. (38). In short, 10 μl aliquots of two-fold serial dilutions of sera were added onto multispot antigen slides containing *P. vivax* Sal 1 blood-stage parasite-infected blood from a *Saimiri* monkey. The slides were incubated in a most chamber at 37°C for 30 min. After washing with PBS, fluorescence isothiocyanate-conjugated goat anti-*Saimiri* IgG antibodies were added. After incubation and washing, antibody titers were determined under a fluorescence microscope.

**Lymphocyte proliferation assays.** To determine the cellular immune responses to the immunogen yP2P30Pv20019, proliferation assays were performed with peripheral blood mononuclear cells (PBMCs) isolated from immunized animals at preimmunization, 4 weeks after the first and second immunization, and 4 weeks after the asexual blood-stage parasite challenge. Proliferation assays were also conducted with spleenocytes 1 week after the blood-stage parasite challenge. For lymphoproliferation assays, blood samples were collected in heparinized tubes and PBMCs were isolated by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) centrifugation. For spleenocytes proliferation assay, spleenocytes were released from the spleen by crushing. After washing with RPMI 1640, 2 × 10^7 PBMCs or spleenocytes in 100 μl of RPMI 1640 supplemented with 5% human AB+ serum and 5% fetal bovine serum were added to 96-well U-bottom culture plates in triplicate. The yP2P30Pv20019 antigen in 100 μl of culture medium (20 μg/ml) was also added to the wells, using phytohemagglutinin (PHA) Sigma Chemical Co., St. Louis, Mo.) at 1:100 dilution and followed by twofold dilutions of sera were added onto multispot antigen slides containing *P. vivax* Sal 1 blood-stage parasite-infected blood from a *Saimiri* monkey. The slides were incubated in a most chamber at 37°C for 30 min. After washing with PBS, fluorescence isothiocyanate-conjugated goat anti-*Saimiri* IgG antibodies were added. After incubation and washing, antibody titers were determined under a fluorescence microscope.

**Statistical analysis.** Data were expressed as geometric means. Differences among groups were compared by Fisher's protected least significant difference test. The association between prechallenge IgG antibody titers and the prepatent period or parasitemia was determined by Pearson correlation analysis. Parasitemia data were analyzed after logarithmic transformation. Significance was declared at P < 0.05.

**RESULTS**

**Prepatent period and parasitemia.** Monkey were immunized three times at 4-week intervals with yP2P30Pv20019 recombinant protein in two different adjuvants: nonionic block copolymer P1005 and alum. No adverse reactions to the immunizations were detected in monkeys immunized with yP2P30Pv20019 or yP2P30Pv20019 in alum during the experimental period. Animals immunized with yP2P30Pv20019 in P1005, however, developed skin sores at the injection sites after the second immunization. One monkey each from the
alum and PBS control groups died from causes unrelated to the study after the second and third immunizations, respectively. All remaining animals were challenged intravenously with 100,000 *P. vivax* Sal I asexual blood-stage parasites. Parasitemia developed in all four animals in the PBS control group (Fig. 1). Of the four animals, three had high parasitemia (accumulated parasite counts of 54,587, 68,536, and 185,916 parasites/μl), and one had a low parasite count (100 parasites/μl) (Table 1). Three of the five animals immunized with *yP2P30Pv20019* in block copolymer P1005 developed low parasitemia (25, 30, and 120 Parasites/μl) and thus were considered to be partially protected; the other two monkeys developed a high parasite counts (93,399 and 291,391 parasites μl). Among the animals immunized with *yP2P30Pv20019* in alum or antigen alone, one animal from each group had a low parasite count (<2,000 parasites/μl), and the remaining animals all developed moderate to high parasitemia (11,703 to 485,947 parasites/μl). In addition, monkeys immunized with *yP2P30Pv20019* in block copolymer P1005 had slightly longer prepatent periods than control monkeys (geometric mean, 12.3 days versus 9.9 days; *P* > 0.05) Table 1).

**IgG antibodies against the *yP2P30Pv20019* recombinant protein.** Levels of total IgG antibodies against the immunogen *yP2P30Pv20019* were measured by ELISA at 2-week intervals, starting 2 weeks after the first immunization (~11 weeks) until the end of the study (6 weeks). Monkeys from the PBS control group had no detectable antibodies after the third immunization (Fig. 2). Animals immunized with the *yP2P30Pv20019* alone had no detectable antibodies after the first immunization. Although antibody titers in monkeys immunized with *yP2P30Pv20019* in alum were low after the first immunization, monkeys immunized with block copolymer P1005 as adjuvant had antibody titers significantly higher than those from all of the three other groups (*P* < 0.05). Antibody titers increased in animals immunized with *yP2P30Pv20019* in block copolymer P1005 or in alum after the second immunization and reached the maximal levels after the third immunization (*P* < 0.05 between the P1005 or alum group and the antigen-alone or PBS group).

Challenge of monkeys with 100,000 asexual blood-stage parasites failed to further boost the titers of antibodies against the immunogen in monkeys immunized with *yP2P30Pv20019* in block copolymer P1005 or in alum, although antibody levels 4 weeks after the challenge were higher than at challenge (week 0). Titers of antibodies against the immunogen from animals immunized with *yP2P30Pv20019* alone or PBS were moderately increased after the challenge and reached higher levels 6 weeks after the challenge. However, antibody levels from animals immunized with *yP2P30Pv20019* in block copolymer P1005 or alum were still significantly higher than in those immunized with antigen alone or PBS at challenge and at 2, 4, and 6 weeks after the challenge (*P* < 0.05).

**IgG antibodies against asexual blood-stage parasites.** Titers of IgG antibodies against air-dried *P. vivax* asexual blood-stage parasites were determined by IFA 1 week after the third immunization, at challenge, and 2 and 4 weeks after the challenge (Fig. 3). One week after the third immunization or at challenge (5 weeks after the third immunization), animals immunized with *yP2P30Pv20019* in block copolymer P1005 or in alum had higher antibody levels than those immunized with antigen alone or PBS. The difference between the block copolymer P1005 group and the three other groups was significant (*P* < 0.05). Challenge with 100,000 *P. vivax* asexual blood-stage parasites boosted antibody titers in all groups, with the block copolymer P1005 group having the highest titers. The difference between the block copolymer P1005 or alum group and
the antigen-alone or PBS group was significant ($P < 0.05$). This boosting in IFA titers after parasite challenge might be due to specific antibodies or antibodies of other parasite specificities.

**PBMC proliferative responses to the yP\textsubscript{50}P\textsubscript{30}P\textsubscript{v20019} recombinant protein.** To evaluate T-cell responses in the yP\textsubscript{50}P\textsubscript{30}P\textsubscript{v20019} recombinant protein, PBMC proliferation assays were performed with monkey PBMCs collected at preimmunization, 4 weeks after the first and second immunization, and 4 weeks after the asexual blood-stage parasite challenge. As a control, PBMCs were simultaneously stimulated with the mitogen PHA. PBMCs from all monkeys responded to PHA stimulation at SI values ranging from 5 to 45, with unstimulated PBMCs having around 2,500 cpm (data not shown). All monkeys (except one from the antigen-alone group) had no proliferative responses to yP\textsubscript{50}P\textsubscript{30}P\textsubscript{v20019} (SI values ranges from 0.62 to 1.80; $P > 0.05$ among all groups) at preimmunization (Fig. 4A). Four weeks after the first immunization, PBMC proliferative responses yP\textsubscript{50}P\textsubscript{30}P\textsubscript{v20019} started to increase in monkeys immunized with yP\textsubscript{50}P\textsubscript{30}P\textsubscript{v20019} in block copolymer P1005 or alum compared with those in the antigen-alone or PBS group. The difference in SI values between the block copolymer P1005 group and alum, antigen alone, or PBS control group significantly ($P < 0.05$) (Fig. 4B). The SI values of monkeys immunized with yP\textsubscript{50}P\textsubscript{30}P\textsubscript{v20019} in P1005 or alum continued to increase 4 weeks after the second immunization, with the block copolymer P1005 group having the highest proliferative responses (SI ranging from 4.0 to 11.47) (Fig. 4C and Table 1). The difference between the block copolymer P1005 group and the other three groups was significant ($P < 0.05$). Challenge of monkeys with 100,000 asexual blood-stage parasites failed to boost PBMC proliferative responses to the immunogen (Fig. 4D). Although the P1005 and alum groups had significantly higher SI values than the control group, the proliferative response in the P1005 group was actually lower than before the challenge.

**Splenocyte proliferative responses to the yP\textsubscript{50}P\textsubscript{30}P\textsubscript{v20019} recombinant protein.** Splenocyte proliferative responses to the immunogen were also conducted 1 week after the asexual blood-stage parasite challenge. Splenocytes from PBS control monkeys had no responses to the stimulation of yP\textsubscript{50}P\textsubscript{30}P\textsubscript{v20019} recombinant protein (mean SI of 0.55 to 1.07, with counts per minute not shown).

![FIG. 2. Levels of IgG antibodies against the yP\textsubscript{50}P\textsubscript{30}P\textsubscript{v20019} recombinant protein in sera from control monkeys (PBS) and monkeys immunized with yP\textsubscript{50}P\textsubscript{30}P\textsubscript{v20019} (Ag [antigen] only), yP\textsubscript{50}P\textsubscript{30}P\textsubscript{v20019} in block copolymer (P1005), or yP\textsubscript{50}P\textsubscript{30}P\textsubscript{v20019} in alum (Alum) three times at 4-week intervals (weeks −13, −9, and −5) and challenged at week 0. Group geometric means are shown.](http://iai.asm.org/)
minute ranging from 769 to 1,439) (Fig. 5). In contrast, splenocytes from monkeys immunized with yP2P30PV20019 in P1005 or alum responded to the immunogen stimulation with average SI values of 4.19 and 4.38, respectively. The difference between the block copolymer or alum groups and the PBS control group was significant ($P < 0.05$).

**Association of partial protection with IgG antibody production.** To assess the role of IgG antibodies in protection, the association between partial protection and IgG antibody levels measured by ELISA and IFA was analyzed. Monkeys (SI-1024, -2126, -2053, -1021, -1069, and -2142) partially protected (with peak parasitemia lower than 1,000/$\mu$L) from the asexual blood-stage parasite challenge had significantly higher prechallenge IgG antibody levels than those unprotected when IgG antibody titers were determined by ELISA (geometric mean of 147,327.7 versus 2,775.1; $P < 0.05$). Likewise, there was also a significant positive correlation between the prepatent period and prechallenge IgG antibody levels by both ELISA and IFA ($r = 0.64$ and 0.63, respectively; $P < 0.05$) (Fig. 6) and a significant negative correlation between accumulated parasitemia and prechallenge IgG antibody titers ($r = -0.59$ for ELISA and $-0.48$ for IFA; $P < 0.05$).

**DISCUSSION**

We have assessed the immunogenicity of a yeast-expressed recombinant protein containing the C-terminal 19-kDa fragment of *P. vivax* MSP-1 and two T-helper epitopes of tetanus.
toxin and have evaluated this recombinant protein formulated in human usable and potentially human-usable adjuvants, alum and block copolymer P1005, for the ability to induce protective immune response in a nonhuman primate malaria model system. Our results showed that Saimiri monkeys immunized three times with the C-terminal 19-kDa recombinant protein formulated in block copolymer P1005 produced titers of IgG antibody response against both the immunogen and asexual blood-stage parasite antigens higher than those immunized with the C-terminal 19-kDa antigen in alum or antigen alone. These monkeys also had significantly higher PBMC proliferative response than PBS control animals when stimulated with the immunogen 4 weeks after the second immunization. Upon challenge with 100,000 asexual blood-stage parasites, monkeys immunized with the C-terminal 19-kDa recombinant antigen in block copolymer P1005 had somewhat longer prepatent periods than control monkeys. Three of the five monkeys in this group developed very low blood-stage parasite infections and thus were partially protected.

Both humoral and cellular immune responses were induced by vaccination with the recombinant 19-kDa protein. Monkeys immunized with the recombinant antigen in P1005 or alum had high titers against the immunogen as well as proliferative responses. Antibody production and proliferative responses were the highest in animals immunized with the 19-kDa antigen in P1005, probably as a result of the potent stimulatory effect of P1005 to both humoral and cellular immunity (43). The protective immunity induced by vaccination, nevertheless, appears to be mediated in part by antibodies. Monkeys with higher IgG antibody titers at the challenge had longer prepatent periods and lower peak and accumulated parasite counts than those with lower IgG antibody titers. Studies with rodent malaria P. yoelii also showed that protection against blood-stage infection after immunization of mice with the C-terminal domain of P. yoelii MSP-1 was partially mediated by antibodies since passive transfer of immune sera or purified IgG protected some naive mice against lethal disease (28). Factors other than antibodies probably also played a role in protection against malaria, because one partially protected monkey (SI-1069) had not titers of antimalaria antibodies.

Adjuvants appear to play a role in protective immunity induced by MSP-1. Previous studies with the C-terminal 42- or 19-kDa fragment of P. falciparum and P. cynomolgi MSP-1 showed that monkeys immunized with the C-terminal fragment were partially protected against blood-stage parasite challenge when the immunogen was formulated in Freund’s complete adjuvant (7, 26, 32). However, when liposome and alum were used as adjuvants, monkeys were not protected against blood-stage parasite challenge (5). In agreement with previous studies (5, 12), results of this study with the 19-kDa fragment of P. vivax showed that alum, the only approved human-usable adjuvant, failed to induce a protective immune response against blood-stage parasite challenge following immunizations, al-
though it induced moderate levels of IgG antibodies. In contrast, when the potentially human-usable adjuvant block copolymer P1005 was used as adjuvant, the recombinant 19-kDa MSP-1 antigen induced a protective immune response in a nonhuman primate malaria model. Our previous studies showed that mice immunized with the C terminus of P. vivax MSP-1 formulated in block copolymer P1005 generated high humoral and cellular immune responses comparable to those for mice immunized with the C terminus in Freund’s complete adjuvant (46). Furthermore, monkeys immunized with a P. vivax circumsporozoite protein-based multiple-antigen construct formulated in P1005 were protected against sporozoite challenge (47). Taken together, these findings indicate that the block copolymer P1005 is an effective adjuvant. Its usage in human malarial vaccine, however, has to be tested further in laboratory animals because of the development of skin sores at the injection sites seen in this study. Perhaps an aqueous phase rather than the water-in-oil emulsion of the adjuvant should be used, because P1005 in aqueous solution has been shown recently to be safe in humans (43). The partial protection induced by immunization with 19-kDa MSP-1 antigen of P. vivax in Saimiri monkeys observed in this study is lower than that recently achieved by immunization against P. cynomolgi in toque monkeys with 42- or 19-kDa MSP-1 antigen (32). In the latter, close to complete protection was obtained by immunization with baculovirus-expressed P. cynomolgi MSP-1 antigens in Freund’s adjuvant. This difference is likely the result of differences in model systems and/or adjuvants. Development of immunity against P. cynomolgi in toque monkeys is rapid; thus, animals recovered from primary infection appear refractory to subsequent infections. In contrast, development of immunity against P. vivax in Saimiri monkeys is a gradual process; animals that recover from primary infection are not protected against subsequent infections.

Further investigations, however, are needed to validate the efficacy of this vaccine. These studies should include adjuvant controls as well as large number of animals per group to minimize the effect of variations in parasitemia and the prepatent period. Subsequent studies should also investigate the protective efficacy of this vaccine in intact rather splencotomized animals. Notwithstanding these issues, results of this preliminary study suggest that the C terminus of MSP-1 is highly immunogenic and may offer some protection against P. vivax blood-stage parasites.

ACKNOWLEDGMENTS

We thank Ae M. Sakchou, Carla L. Morris, Robb C. Reed, and personnel at the Scientific Resources Program, National Center for Infectious Diseases, CDC, for technical assistance.

This work was supported in part by USAID IAA 963-9001-G-00-6-540-00.

REFERENCES


