Baculovirus Merozoite Surface Protein 1 C-Terminal Recombinant Antigens Are Highly Protective in a Natural Primate Model for Human Plasmodium vivax Malaria

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A successful anti-blood stage malaria vaccine trial based on a leading vaccine candidate, the major merozoite surface antigen-1 (MSP1), is reported here. The trial was based on Plasmodium cynomolgi, which is a primate malaria parasite which is highly analogous to the human parasite Plasmodium vivax, in its natural host, the toque monkey, Macaca sinica. Two recombinant baculovirus-expressed P. cynomolgi MSP1 proteins, which are analogous to the 42- and 19-kDa C-terminal fragments of P. falciparum MSP1, were tested by immunizing three groups of three animals each with either p42, p19, or both together. The vaccines were delivered subcutaneously in three doses at 4-week intervals with complete and incomplete Freund's adjuvants. Very high antibody titers were obtained against both vaccinating antigens as measured by enzyme-linked immunosorbent assay (106 and 108) and against whole parasites as measured by indirect immunofluorescence assay (≥104), achieving, in most animals, a 10-fold increase from the first to the last immunization. A blood stage challenge with P. cynomolgi parasites led, in three adjuvant-treated and three naive control animals, to blood infections which were patent for at least 44 days, reaching peak densities of 0.6 and 3.8%, respectively. In contrast, all except one of the nine animals in the three vaccinated groups were highly protected, showing either no parasitemia or transient parasitemias which were patent for only 1 or 2 days. When the three p19-vaccinated monkeys were rechallenged 6 months later, the protective efficacy was unchanged. The success of this trial, and striking analogies of this natural host-parasite system with human P. vivax malaria, suggests that it could serve as a surrogate system for the development of a human P. vivax malaria vaccine based on similar recombinant analogs of the P. vivax MSP1 antigen.
out with this natural nonhuman primate system involving transmission-blocking immunity (34), relapses (unpublished data), and pathogenesis (33) have found direct application to human P. vivax malaria (31). P. cynomolgi and P. vivax MSP1 C-terminal recombinant proteins produced in the baculovirus expression system are highly homologous and appear to reproduce the physical and antigenic characteristics of the native proteins (22, 28). This report presents a highly successful vaccine trial with the toque monkey, showing excellent immunogenicity and protective efficacy of the P. cynomolgi MSP1 recombinant proteins.

MATERIALS AND METHODS

Animals. Adult toque monkeys (M. sinica sinica) were captured from their natural habitats in the dry zone of Sri Lanka and were quarantined in the animal house for 1 month prior to experimental use. All animals were screened for the presence of a current malaria infection by microscopic examination of thick and thin blood films and for previous exposure to malaria by detection of serum antiplasmodial antibodies by the indirect immunofermunoassay (IFA). An animal was considered naive with respect to malaria when thick blood films failed to detect parasites for 7 consecutive days and anti-P. cynomolgi antibodies were undetectable. We have shown that such animals are free of subpatent malaria infections because they are incapable of transmitting malaria infections to other naive recipients by blood passage (20, 32).

Recombinant immunogens. P. cynomolgi MSP1 recombinant proteins corresponding approximately to the P. falciparum MSP1 42- and 19-kDa C-terminal processing fragments (28, 29) were produced in the baculovirus expression system and purified by immunoaffinity chromatography with monoclonal antibodies as described previously (22).

Immunization of animals. Fifteen malaria-naive animals comprising five groups of three animals each, matched by sex and weight (Table 1), were used for the trial. Animals in the first three groups (groups I, II, and III) were vaccinated with either p42, p19, or a combination of the two antigens, respectively, and those in group IV were vaccinated with normal saline; group V served as unvaccinated control. Each inoculation consisted of 100 μg of the p19 antigen administered subcutaneously in three doses at 4-week intervals. Each dose was suspended in 0.5 ml of normal saline and emulsified in an equal volume of adjuvant consisting of Freund’s complete adjuvant (FCA) and Freund’s incomplete adjuvant (FIA) in proportions of 1:1 (first immunization) and 1:4 (second immunization). Only FIA was used for the third and last immunization. Immunized control animals in group IV were administered normal saline with the same adjuvant protocol. No clinically adverse reactions to vaccination were evident in any of the animals, even at the sites of subcutaneous vaccine injection. Serum samples were taken prior to the first immunization and 2 to 3 weeks after each immunization.

Challenge infections and evaluation of protective efficacy. Animals in all five groups were challenged 4 weeks after the third immunization with an inoculum of 2 × 10⁷ asexual blood stage P. cynomolgi cypræensis parasites obtained from a donor monkey. Parasites are maintained in the laboratory by blood and mosquito passage and cryopreservation (32, 34). Commencing from the fourth day after challenge, all 15 animals were screened for asexual blood stage parasites by microscopic examination of Giemsa-stained thick and thin smears prepared from an earclip. Blood film examinations were conducted daily for 6 weeks and weekly up to 8 weeks postchallenge.

On the 30th day of patency, blood samples from all animals were analyzed by PCR with standard methods and primers for small-subunit rRNA derived from P. vivax sequences (40). These primers amplify P. cynomolgi DNA with equal efficiency, as expected from the high degree of sequence homology between the two species (reviewed in reference 28). A second blood challenge with 10⁸ parasites was given to animals in the p19-vaccinated (group II) and adjuvant-treated control (group IV) groups 6 months later.

ELISA. Ninety-six-well microtiter plates (Nunc, Roskilde, Denmark) were coated with 0.1 μg of either p19 or p42 antigen per ml and incubated at 4°C overnight, and enzyme-linked immunosorbent assays (ELISAs) were carried out as previously described (37) with horse-radish peroxidase-conjugated goat anti-human immunoglobulin (1:1,000 dilution) (DAKO Immunoglobulins, Copenhagen, Denmark). The optical density was read at 492 nm and the ELISA end point was taken as the highest serum dilution which gave an optical density greater than twice that of control sera.

IFA. P. cynomolgi schizont-infected erythrocytes were purified from infected blood by a Percoll density gradient method as described previously for P. vivax (23). The infected-erythrocyte suspension was spotted onto 12-well antigen slides, air dried, and stored at −70°C. The IFA was performed as previously described (42) with fluorescein isothiocyanate-conjugated goat anti-human immunoglobulin G (1:1,000 dilution) (DAKO Immunoglobulins, Copenhagen, Denmark). The optical density was read at 492 nm and the ELISA end point was taken as the highest serum dilution which gave an optical density greater than twice that of control sera.

RESULTS

Immunogenicity. Both the recombinant p19 and p42 antigens, either alone or in combination, were highly immunogenic, since animals in all three MSP1-vaccinated groups
developed very high antibody titers against the vaccinating antigens as detected by ELISA (10^6 and above) and against *P. cynomolgi* schizont-infected erythrocytes as measured by IFA (≥10^5) (Table 1). Although antibody titers were already quite high after a single injection of the recombinant antigen, these titers increased in postimmunization sera in most animals by about 10-fold or more from the first to the last injections (Fig. 1). IFA titers of these sera also showed a similar increase through consecutive immunizations (data not shown).

**Protection against parasite challenge.** Following challenge, adjuvant-treated control animals of group IV developed *P. cynomolgi* parasitemias similar to those in the unvaccinated controls. All animals in both groups had patent infections for 39 to 54 days, although the parasite densities in the adjuvant-treated controls were significantly lower than those in the unvaccinated controls (*P = 0.0006*). These adjuvant-treated controls also experienced lower peak parasitemias (0.1 to 0.6% than unvaccinated controls (0.4 to 3.8%) (Fig. 2A; Table 1), indicating some effect of adjuvant alone. In contrast, the three animals immunized with p19 (group II) developed either no parasitemia at all (T429) or a transient infection of 0.002% which was patent for either 1 (T427) or 2 (T426) days (Fig. 2C; Table 1). One animal in the p42-vaccinated group (group I, animal T435) developed a parasitemia that peaked at 0.06% and persisted for 17 days. The parasite densities from day 5 to 32 of challenge infections in each of the three vaccinated groups were significantly lower than those in both the adjuvant-treated control group and the unvaccinated control group (*F = 23.7788; *P < 0.0001*).

PCR analysis was performed on blood samples from all animals 34 days after challenge in order to detect subpatent parasitemias. A positive PCR was obtained for all control animals (groups IV and V), while no PCR signal was observed in the vaccinated animals (groups I to III), indicating that any low-grade, microscopically subpatent blood infection was below the limits of detection by PCR (data not shown). All six control animals continued to have a patent parasitemia until day 44 after challenge; four of the six animals then had cleared their blood parasitemias, but the other two (one in each group) still had parasitemias of 0.004%. On the 68th day after challenge, all animals (vaccinated and control) were treated with a 3-day curative regime of chloroquine (25 mg per kg of body weight) administered intramuscularly to eliminate any remaining blood infection.

To examine the state of protective immunity 6 months after the immunization and the first challenge, a second blood challenge of 10^6 parasites but otherwise identical to the first was given to three groups, as follows: (i) the three animals vaccinated with p19 (group II), two of which had experienced a transient blood infection during the first challenge; (ii) the three animals in the adjuvant-treated control group (group IV) that were unprotected during the first challenge and therefore developed a complete blood infection; and (iii) a new control group of two naive unvaccinated animals. The two naive animals developed a normal blood infection with a peak 0.8% parasitemia which was still patent on day 20 after challenge, when daily screening was discontinued (Fig. 3A). In contrast, all animals in the other two groups were very well protected after the second challenge, with only one of the three animals in each group (T426 and T425) developing transient parasitemias of 0.008%, which were patent for only 1 day (Fig. 3B and C). These findings indicate that the animals vaccinated with p19 and only minimally stimulated by the first challenge infection, which resulted in either very low or no (T429) detectable infection, were able to resist a second challenge infection as well as the adjuvant-treated controls, which were protected by exposure to a much higher complete blood infection during the first challenge. Western blotting was performed with sera taken from both groups of animals, just before the second challenge, using schizont extracts of *P. cynomolgi*. While the sera of the adjuvant-treated control animals reacted with parasite antigens with a wide range of molecular weights, the sera of all three p19 vaccinees reacted strongly and only

![FIG. 1. ELISA end point titers of all pre- and post-immunization sera against p42 (A) and p19 (B) recombinant proteins.](http://iai.asm.org/)

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with the MSP1 processing products on the Western blot (data not shown). These p19 vaccinees also had very high titers of antibodies (10^6) against the recombinant p19 antigen when tested by ELISA. These findings indicate that the continued protected state in the p19 group must have been mediated by this sustained or boosted anti-MSP1 p19 immune response.

**DISCUSSION**

Vaccination with the baculovirus-expressed p42 and p19 C-terminal P. cynomolgi MSP1 recombinant proteins resulted in a very high antibody response and very effective protection against a challenge infection in eight of nine animals, with an apparently sterile immunity induced in two. The low transient blood parasitemias of challenge infections seem to have been completely cleared, since parasite DNA could not be detected by PCR 34 days after challenge. The three animals in the p19-vaccinated group were also able to resist a second challenge 6 months later.

The high degree of protection elicited in this trial could be due to the nature of the recombinant proteins themselves, which were produced in a higher-order eukaryotic system and appear to reproduce the complex conformational structure of the native protein. We have, in fact, shown that the reactivity of human antisera from P. vivax-immune individuals is completely dependent on the integrity of reduction-sensitive conformational epitopes present in the p19 recombinant protein (22).

It is noteworthy that the smaller MSP1 p19 antigen containing only the two EGF domains conferred at least as good protection as the larger p42 antigen, suggesting that this part of the molecule is sufficient to generate protective immunity. This clearly indicates that the p19 antigen must have its own functional B- and T-cell epitopes that mediate a strong effective immune response without the addition of extraneous non-MSP1 peptides. We have previously shown that the baculovirus-expressed recombinant MSP1 p42 and p19 antigens have distinct physical properties and appear to display epitope subsets that are specific to each (22). In addition, the p42 protein appears to have a hypervariable region which may play a role in immune evasion, assuming that it is the focus of an effective antiparasite immune response (28). The presence of this region in a subunit vaccine might therefore be detrimental in a malaria-endemic environment where the risk of exposure to polymorphic variants in the hypervariable region may render vaccination with a single variant less effective.

The potent protective immunity observed in this trial might have been contingent upon the exceptionally high antibody titers that were elicited in the vaccinated animals by using Freund’s adjuvant. Indeed, the serum of the least protected vaccinee (T435) showed the slowest rise in ELISA titers against both the recombinant antigens, even though the titers were still quite high (10^6). In the primate P. falciparum trials involving recombinant MSP1 antigens, the protection observed did not appear to be dependent on antibody titers (3, 26). However, if this level of vaccine-induced protection does indeed require the presence of such high antibody titers, then the challenge ahead for preparation of an analogous human P. vivax vaccine will be to replace the potent Freund’s adjuvant. Indeed, parasitemias after challenge in the Freund’s adjuvant-treated controls were generally somewhat lower than those in the unvaccinated controls, suggesting that this strong adjuvant

![FIG. 2. Courses of blood parasitemia following challenge infection with P. cynomolgi in naive unvaccinated (group V) and adjuvant-treated control (group IV) animals (A) and in animals immunized with p42 (group I) (B), with p19 (group II) (C), and with both p42 and p19 (group III) (D).](http://iai.asm.org/)
Protection as complete as that elicited in this trial has rarely, if ever, been achieved in primates with a recombinant subunit malaria vaccine against blood stage parasites. These results are now being confirmed in a trial with larger numbers of animals. Consistent success has, however, recently been achieved by immunization with baculovirus-expressed recombinant products of this antigen, both in *P. vivax* with MSP1 p42 and p19 in a nonsplenectomized squirrel monkey (*Saimiri sciureus boliviensis*) model (1a) and in *P. falciparum* with MSP1 p42 in *Aotus* monkeys (3), even though both used FCA and FIA as adjuvants. A recent successful clinical trial of a preerythrocytic stage recombinant malaria vaccine in conjunction with an oil-in-water adjuvant containing monophosphoryl lipid A and QS21 (41) has considerably heightened the prospects for replacing Freund’s adjuvant for human use.

The results of this trial have demonstrated that a high degree of protection against a malaria blood infection can be achieved in this natural primate host-parasite system by vaccinating with a recombinant subunit vaccine. Two points need to be considered in assessing the relevance and stringency of this model for vaccine evaluation. First, in the course of an uninterrupted challenge infection of *P. cynomolgi* in the toque monkey, parasitemias peak at less than 5% and last for at least 44 days, and probably more than 58 days, after which they are self-cured. These characteristics are similar to those of untreated *P. vivax* infections in human volunteers, which reached parasitemias of less than 10% and remained patent for 60 days or more prior to being self-cured (4).

Second, a complete *P. cynomolgi* blood infection in the adjuvant-treated control animals following the first challenge led to almost complete protection against a second challenge with the homologous strain 6 months later. Although this might suggest that protective immunity is too easily achieved in this system for it to be a valid immunization model for human malaria, two previous vaccine trials that were conducted with this system, using some of the same antigens under different conditions, failed to provide any protection, for a variety of possible reasons (36a). However, more important, it is likely that protection against a homologous strain of *P. vivax* in humans can also be induced by a single blood infection provided that the infection is allowed to run its natural course without drug treatment. Evidence for this comes from reports of early investigators who studied malaria infections which were therapeutically induced in neurosyphilitic patients (2, 24). Untreated *P. vivax* infection gave rise to complete clinical protection against a subsequent challenge with the identical strain but not with a different strain of the same species (2); since no parasitological observations were made in these studies, it is not possible to determine the extent to which antiparasite protection was achieved by these infections. Similar studies reported by Jeffery (24), in which all of the induced infections were drug cured, did not give rise to such a striking immunity, implying that the early termination of infection interferes with the development of immunity. The results of these early experiments also emphasize that the difficulty in acquiring natural protection against malaria must be largely attributable to polymorphism in target antigens or epitopes of protective immunity among natural parasite populations.

To test a malaria vaccine destined for use in humans, an analogous natural primate malaria may be superior to artificial experimental systems based on the human malaria parasites themselves in unnatural hosts. Nevertheless, the real relevance of the results reported here will be determined only by clinical trials with the *P. vivax* MSP1 recombinant analogs. In the meantime, however, this system will serve to address several important questions raised by this promising trial. These in-

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**FIG. 3.** Courses of blood parasitemia following the first and second challenge infections with *P. cynomolgi* in naive control animals (A), in adjuvant-treated control animals (group IV) (B), and in p19-vaccinated animals (group II) (C).
clude, principally, the need for testing candidate vaccine antigens under statistically valid conditions and the search for effective adjuvants which are acceptable for human use.

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