REPEATED INFECTION OF AOTUS MONKEYS WITH PLASMODIUM FALCIPARUM INDUCES PROTECTION AGAINST SUBSEQUENT CHALLENGE WITH HOMOLOGOUS AND HETEROLOGOUS STRAINS OF PARASITE

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Abstract. We evaluated repeated blood-stage infections with Plasmodium falciparum in eight Aotus lemurinus lemurinus monkeys. Over the course of seven infections with 10⁶ P. falciparum (the Vietnam Oak Knoll [FVO] strain), the pre-patent period lengthened from 8.2 to 30.8 days; the peak parasitemia decreased from 4.5 × 10⁴ to 0 parasites/μl (Challenges 6 and 7), and the requirement for treatment decreased from 100% to 0% (Challenges 3 to 7). Five weeks after the seventh FVO challenge, the eight immune and three naïve monkeys received 10⁴ parasitized erythrocytes infected with P. falciparum (CAMP strain). The three control animals experienced uncontrolled parasitemias reaching between 4.8 and 7.7 × 10⁵ parasites/μl (pre-patency = 6.3 days) and all required drug treatment; six of the eight immune monkeys became parasitemic (pre-patency = 8.8 days), but self-cured. Two of three of the monkeys having the greatest reductions in hematocrit (50–60%) also had the highest parasitemias (10⁴–10⁵ parasites/μl) before self-curing. Repeated homologous infections induced sterile immunity to homologous challenge; during heterologous challenge the monkeys developed clinically relevant, but not life-threatening, parasitemias and anemia.

INTRODUCTION

We believe that studying the response of Aotus monkeys to repeated infections with lethal strains of Plasmodium falciparum may provide important insights into the requirements of and potential for erythrocytic stage P. falciparum vaccines. We therefore studied the response to repeated infections with the highly virulent Vietnam Oak Knoll (FVO) strain of P. falciparum,¹ and the response of monkeys immune to repeated challenge with FVO to challenge with a different strain of P. falciparum, CAMP. Our goals were to determine 1) how many exposures were required before the monkeys were able to control the parasitemia, 2) if the monkeys developed sterile protective immunity, and 3) if monkeys immune to challenge with FVO were susceptible to challenge with CAMP. The results clearly demonstrate that by their third exposure to P. falciparum, the monkeys developed immunity which led to cure without treatment, and that by the sixth exposure they developed sterile protective immunity against a single strain of P. falciparum. Furthermore, when such completely immune monkeys were challenged with another strain of P. falciparum, they developed parasitemias at essentially the same rate as controls, developed a level of parasitemia that would cause illness in humans, and three of the eight monkeys experienced a decrease of ≥ 50% in their hematocrits. However, they then controlled the parasitemias and did not develop the level of parasitemia or anemia associated with severe disease in humans.

MATERIALS AND METHODS

Monkeys. Panamanian adult (male and female) Aotus lemurinus lemurinus (karyotype VIII or IX) monkeys² were maintained in the animal facility of the Gorgas Memorial Institute in Panama City, Republic of Panama. Aotus l. lemurinus monkeys were obtained in western Panama. Upon arrival at the laboratory, each animal was given a physical examination, weighed and sexed, identified by a metal neck tag with an accession number, administered thiabendazole orally for treatment of endoparasites (100 mg base/kg) and vaccinated against Herpes simplex, Herpes tamarinus (New England Regional Primate Research Center, Southborough, MA) and Klebsiella pneumoniae³. The animals were housed and cared for as previously described.³ About one month prior to the first FVO challenge described here, the animals were splenectomized.

 Parasite strains. Immunity to parasite challenge was established using the Vietnam Oak Knoll (FVO) strain of P. falciparum¹. The heterologous strain challenge was performed using the CAMP strain of P. falciparum which was originally isolated in Malaysia⁴. Challenge protocol. The monkeys described in this study received their first challenge with P. falciparum (FVO) either as naïve controls in other studies (n = 2) or after receiving one of three first generation monovalent malaria vaccines (n = 6). Once these studies were completed, the animals were reallocated to this study and re-challenged (Figure 1). None of the animals had been infected with P. falciparum prior to the first FVO challenge described here. The animals received their first FVO challenges between October 1994 and August 1995, and then six more FVO challenges approximately every five months until all had received seven FVO challenges. All animals received their seventh FVO challenge simultaneously. Five weeks after the seventh FVO challenge and two weeks prior to challenge with the heterologous CAMP all animals were treated orally once a day for five days with 50 mg/kg quinine and 6 mg/kg doxycycline to eliminate any occult FVO parasitemia. All then received the heterologous CAMP strain challenge 10 days after treatment was concluded. All challenges described in this study were performed by the IV injection via the saphenous vein of 10⁴ parasitized Aotus erythrocytes.

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Blood films. During periods of surveillance, thick blood films were prepared with blood taken from a marginal ear vein by lancet venipuncture. The slides were Giemsa stained and the parasites enumerated using the method of Earle and Perez. A negative parasitemia was recorded if no parasites were seen after 100,000 fields (approximately 0.65 μl of blood) were examined.

Hematocrit. Hematocrits were monitored on days 10, 14, 17 and 21 post-CAMP challenge. Fifty μl of blood was drawn into a heparinized capillary tube, then centrifuged in a microhematocrit centrifuge for five minutes prior to reading.

Treatment. Monkeys were treated if parasitemia reached 400,000 parasites/μl, or for clinical deterioration. Treatment was a single oral dose (20 mg/kg) of mefloquine.

Immunization status. As part of other studies conducted prior to this study, 6/8 of the monkeys received immunizations DNA plasmids prior to the FVO infection. Three animals (monkeys 12756, 12759, 12757) received three doses of a P. falciparum (FVO strain) Merozoite Surface Antigen 1C (PfMSP-1C) DNA vaccine (Kumar S, unpublished data), an antigen expressed by blood stage parasites. Two other animals (monkeys 12765, 12763) received three doses of a P. falciparum PfS25 DNA vaccine, an antigen expressed on parasite gametes. One animal (monkey 12730) received three doses of P. falciparum (FVO strain) Apical Merozoite Antigen-1 (PfAMA-1) DNA vaccine (Aguiar J, unpublished data). Table 1 outlines the immunization regimens.

Statistics. Statistical tests were performed using SPSS for Windows 8.0 (SPSS, Chicago, IL) and the STATCALC module in EpInfo (Centers for Disease Control and Prevention, Atlanta, GA). Power calculations were performed in SamplePower 1.00 (SPSS).

Animal use. The experiments were conducted according to the principles set forth in the “Guide for the Care and Use of Laboratory Animals,” Institute of Laboratory Animal Resources, National Research Council (Department of Health and Human Services, National Institutes of Health Publication 86-23, 1985).

RESULTS

Parasitemia. After the first and second challenge with FVO, all monkeys became parasitemic. After the third, fourth and fifth challenges, 6/8, 2/8 and 4/8 respectively became parasitemic. None of the animals became parasitemic.

Table 1

<table>
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<tr>
<th>Monkey no.</th>
<th>Sex</th>
<th>Immune status</th>
<th>FVO1 challenge</th>
<th>FVO2 challenge</th>
<th>FVO3 challenge</th>
<th>FVO4 challenge</th>
<th>FVO5 challenge</th>
<th>FVO6 challenge</th>
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<td></td>
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<td>PP</td>
<td>PC</td>
<td>PP</td>
<td>PC</td>
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<td>64</td>
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<td>8</td>
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<td>106</td>
<td>12.6</td>
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<td>578*</td>
<td>7</td>
<td>6.3</td>
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* treated with three doses of mefloquine daily for three days (20 mg/kg).
† treated with a single dose of mefloquine (20 mg/kg).

PfS25 = P. falciparum sexual stage antigen 25, 500 μg plasmid DNA ID administered on Weeks −8, −5, and −2 prior to the first FVO challenge.
PfAMA-1 = P. falciparum apical membrane antigen-1, 2 mg IM administered on Weeks −9, −6, and −3 prior to the first FVO challenge.
PfMSP-1C = P. falciparum merozoite surface antigen 1C, 500 μg ID administered on Weeks −8, −5, and −2 prior to the first FVO challenge.
PC = peak parasitemia; PP = peak parasitemia; M = male; F = female.
nc = not challenged; GeoMean = geometric mean; PP = peak parasitemia.

FIGURE 1. Eight Aotus monkeys were repeatedly challenged with Plasmodium falciparum, seven consecutive times with the Vietnam Oak Knoll (FVO) strain then once with CAMP strain. Time frames during which the repeated challenges occurred are shown below.
Pre-patency. The pre-patency lengthened after each FVO infection: 8.3 (95% CI: 7.8, 8.7) days after the first infection, 12.6 (11.6, 13.7) days after the second, 19 (9.2, 28.9) days after the third, 22 (18.1, 25.9) days after the fourth, and 31.8 (9.0, 52.5) days after the fifth infection (P = 0.013, one-way ANOVA). The mean pre-patency period after the CAMP challenge was 8.8 (6.7, 10.9) days for the immune monkeys, while the mean pre-patency in the naive control monkeys infected at the same time was 6.3 (5.7, 7.0) days. The difference between the two pre-patency periods in the CAMP challenge (8.8 and 6.3 days) was not significant by t-test (P = 0.160). Our power to detect a true difference of that magnitude with our sample size (8 and 3) was 43%. The mean pre-patency periods for those infections in which animals became parasitemic are shown in Figure 2.

Peak parasitemia. Comparisons of peak parasitemia in the first two challenges are difficult because treatment was not instituted based solely on parasite density in the blood. All eight monkeys received treatment at first infection due to apparently uncontrolled parasitemias of between \(2 \times 10^4\) and \(1.2 \times 10^6\) parasites/µl (geometric mean = 445,409 parasites/µl). At second infection, 8/8 became parasitemic and 3/8 of those self-cured after developing parasitemias ranging from \(2 \times 10^4\) to \(1.8 \times 10^5\) parasites/µl (geometric mean = 106,621 parasites/µl). All monkeys that became parasitemic during FVO challenges 3, 4 and 5 self-cured (geometric mean peak parasitemias = 221, 2 and 2 parasites/µl respectively). Six of eight of the monkeys challenged with CAMP became parasitemic with a geometric mean of 1830 parasites/µl, and none higher than 10,920 parasites/µl. None of the monkeys challenged repeatedly with FVO required drug treatment after the CAMP challenge (Fig 3), and none was parasitemic after day 18. All were followed for 100 days and did not become parasitemic again. On the other hand, all three naive control animals challenged with CAMP required treatment on Day 12 or 13 (Table 1) due to parasitemias ranging between 4.8 and 7.7 \(10^5\) parasites/µl.

Hematocrit. After the CAMP challenge, mean hematocrit for the eight immunized monkeys decreased from 50.6% on Day 10 post-CAMP challenge to 36.5% on Day 17, then stabilized, averaging 36.7% on Day 21 and increasing to 48.5% by Day 31 post-CAMP challenge. The hematocrits of the three naive monkeys also decreased to a low of 38.6% on Day 17 post-challenge, but increased to 47.3% on Day 21 (Table 2). All three of the naive monkeys were treated with mefloquine on Day 12 or 13. The two immune monkeys with the highest parasitemias during the CAMP challenge (monkey 12765 at 9,890 parasites/µl and monkey 12763 at 10,920 parasites/µl) also had the largest decrease in hematocrit referent to the Day 10 baseline during the challenge (59.6% decrease and 51.8% decrease respectively). In spite of these decreases, the hematocrits in both these animals returned to normal by Day 35 (49% and 50% respectively).

Immunization status. The animals were divided into two groups based on their immunization status. Animals designated as “immunized” had received early generation AMA-1 or MSP-1 DNA vaccines while those designated as “not immunized” had received either no immunizations or a Pf25 DNA vaccine, an antigen not known to be expressed by blood stage parasites. No association between immunization status and mean pre-patent period during FVO infections 1 and 2, and during the CAMP infection was noted (independent samples t test, P = 0.36, P = 0.10, P = 0.3, respectively). Calculations to determine our power to detect a difference between these two groups given that a true difference exists indicated that for the first FVO challenge, we had a 0.29 probability of detecting a true difference of 0.5
different between those studies and work reported here include differences in the vaccine evaluation. There was also demonstration of varying degrees of protection from subsequent challenge with the same strain. 

In our studies, repeated challenge with 10⁴ blood stage P. falciparum FVO parasites induced complete resistance to subsequent FVO challenge after 2 (n = 1), 3 (n = 1), 4 (n = 2) or 5 (n = 4) homologous challenges. With the sixth challenge, all eight animals were resistant to infection with 10⁴ infected Aotus erythrocytes to the extent that no parasites were found on thick blood film examination. Resistance to infection was also manifest by a lengthening in the pre-patent period, from an initial 8.25 days to 30 days at the fifth infection (Table 1). Others have reported a similar ability to protect both Aotus and Saimiri monkeys from infection with P. falciparum. In our study, none of the animals required treatment beginning with the third infection. This capacity to self-cure is a significant development. If a vaccine based on a gene sequence from a single clone of P. falciparum led to the development of parasitemia like that found in the monkeys with the third infection, it would have an enormous impact.

<table>
<thead>
<tr>
<th>Monkey no.</th>
<th>Maximum (parasites/µl)</th>
<th>Day*</th>
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<tr>
<td>12749</td>
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<tr>
<td>12943</td>
<td>767250</td>
<td>13‡</td>
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</table>

* day upon which the maximum parasite density occurred.
† the lowest hemocrit recorded for that animal.
‡ treated, mefloquine 20 mg/kg on day noted.
NA = not available. Monkey 12739 died on Day 3. Necropsy revealed an enlarged spleen and liver, but no indication that the death was malaria-related.

### Discussion

Aotus monkeys have been used successfully as one of the few animal models useful in the study of the biology of P. falciparum infection, having first been infected in 1967, and subsequently used extensively in drug treatment and vaccine evaluation. A small number of heterologous challenges have been performed in the past using P. falciparum strains in Aotus monkeys. These studies, performed in other species of Aotus, also showed that previous infection provided protection from subsequent challenge with the same strain. There was also demonstration of varying degrees of protection to heterologous challenge, but a variety of differences between those studies and work reported here include different Aotus species, different P. falciparum strains, and the use of splenectomized animals.

In this study, we have evaluated the ability of repeated blood stage infection with the FVO strain of P. falciparum to induce immunity to further FVO infections, and immunity to infections with a different strain of P. falciparum, the CAMP strain. Although FVO and CAMP were both originally isolated in Indochina, FVO in Vietnam and CAMP in Malaysia, we selected CAMP as the heterologous challenge strain because 1) it, like FVO, is adapted to Aotus, and 2) there is evidence that there are differences between the two strains in antigens thought to be important in immunity to blood stage infection. Specifically, a search of the National Center for Biotechnology Information (NCBI) at The National Institutes of Health was conducted to identify P. falciparum antigens for which the amino acid sequences have been reported for both FVO and CAMP. In region II, which includes the glycoPhorin A binding domain, of erythrocyte binding protein 175 (EBA–175), four of 616 (0.65%) amino acids were non-identical (accession X52524; accession AAU27390). The reported sequences for FVO and CAMPAMA–1 were 3.37% non-identical (21 of 622 residues) (accession M58545, Aguai and Hoffman, direct submission to NCBI, accession U84348). The FVO and CAMP strains possess different alleles of MSP-1; at the amino acid level, these alleles have 60% identity, 73% identity when including synonymous changes (accession X03831; Louis-Wileman, Shi, Collins and Lal, direct submission to NCBI, accession L20092). In addition, RFLP analysis of FVO and CAMP showed length differences in ring-infected erythrocyte surface antigen (RESA). Phenotypic differences between the two strains in infection pattern, gametocyte production, and auto-agglutination have also been reported.

In our studies, repeated challenge with 10⁴ blood stage P. falciparum FVO parasites induced complete resistance to subsequent FVO challenge after 2 (n = 1), 3 (n = 1), 4 (n = 2) or 5 (n = 4) homologous challenges. With the sixth challenge, all eight animals were resistant to infection with 10⁴ infected Aotus erythrocytes to the extent that no parasites were found on thick blood film examination. Resistance to infection was also manifest by a lengthening in the pre-patency period, from an initial 8.25 days to 30 days at the fifth infection (Table 1). Others have reported a similar ability to protect both Aotus and Saimiri monkeys from infection with P. falciparum. In our study, none of the animals required treatment beginning with the third infection. This capacity to self-cure is a significant development. If a vaccine based on a gene sequence from a single clone of P. falciparum led to the development of parasitemia like that found in the monkeys with the third infection, it would have an enormous impact.
on the morbidity and mortality of malaria, especially if effective against a variety of strains. We therefore cross-challenged the monkeys with the CAMP strain. While all the monkeys controlled their CAMP parasitemias (unlike their first two FVO challenges, Table 1), six of eight did develop patent parasitemias (unlike their sixth and seventh FVO challenges). Furthermore, by fifth FVO infection, the mean pre-patent period had gone from 8.2 days with first infection to 30 days, but when these animals were challenged with CAMP the pre-patent period was reduced to 8.8 days. In addition, three of the animals had at least 50% decreases in hemocrit (Table 2). Nonetheless all CAMP-infected monkeys controlled their parasitemias. Geometric mean peak parasitemia after the first FVO infection was 445,000 parasites/μl compared with 1,825 parasites/μl for the CAMP infection (\(P < 0.001\), t-test); the latter did not require treatment.

The fact that all of the animals developed sterile protective immunity to homologous challenge, and controlled the heterologous challenge parasitemia without treatment provides an important yardstick for vaccine developers. As stated above, if a vaccine did this, it would eliminate much of the severe morbidity and mortality of malaria. However, such a vaccine would not be optimal for non-immune travelers. If the effect of prior immunization is unwise because of the statistical non-significance of the differences, and the lack of statistical power to resolve meaningful differences.

In summary, repeated infection with one strain of \(P. falciparum\) (FVO) led to sterile protective immunity against the same strain, and provided significant protection from overwhelming parasitemia in a subsequent challenge with another strain (CAMP). This protection did not, however, extend to protection from anemia. Those animals with the highest parasitemias after CAMP challenge, while self-curing their infections, experienced very significant decreases in hematocrit. Further characterization of the pathogenesis of the anemia will be important.

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