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 10⁴ 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 × 10⁷ 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 naive 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 × 10⁷ 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 (10² 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 Gorgias 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 varicellaeus (New England Regional Primate Research Center, Southborough, MA) and Klebsiella pneumoniae.³ The animals were housed and cared for as previously described.¹ About one month after arrival, each monkey was tattooed with its identification number and a thick blood film examined to exclude naturally occurring plasmodial infections. The animals remained in quarantine for a minimum of 90 days before being transferred to areas devoted to housing monkeys for malaria studies. The weight of the monkeys when inoculated ranged from 700 to 800g. All monkeys used in these experiments were wild caught adult monkeys and had no history of experimental infection with Plasmodium. None of monkeys was splenectomized.

Parasite strains. Immunity to parasite challenge was established using the Vietnam Oak Knoll (FVO) strain of P. falciparum.¹ The heterologous strain challenge was performed using the CAMP strain of P. falciparum which was originally isolated in Malaysia.⁴

Challenge protocol. The monkeys described in this study received their first challenge with P. falciparum (FVO) either as naive 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.
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 600x 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 first FVO infection. Three animals (monkeys 12756, 12759, 12757) received three doses of a *P. falciparum* (FVO strain) Merozoite Surface Antigen 1 (PMSP-1<sub>142</sub>) DNA vaccine (Kumar S, unpublished data), an antigen expressed by blood stage parasites. Two other monkeys (monkeys 12765, 12763) received three doses of a *P. falciparum* PfSP25 DNA vaccine, an antigen expressed on parasite gametes. One animal (monkey 12730) received three doses of *P. falciparum* (FVO strain) Apical Merozoite Antigen-1 (PIAMA-1) DNA vaccine (Aguir 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.

**Highest recorded parasitemia (parasites/μl blood) and length of pre-patent period (days) for each monkey repeatedly challenged with the Vietnam Oak Knoll (FVO) strain of *Plasmodium falciparum* then challenged with the CAMP strain of *P. falciparum*. Three naive control animals challenged with CAMP strain only are included. Antigens used in any immunizations given prior to the start of the challenges are noted.**

<table>
<thead>
<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>
<th>FVO7 challenge</th>
<th>CAMP challenge</th>
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<tbody>
<tr>
<td>12765</td>
<td>M</td>
<td>Pf25</td>
<td>840*</td>
<td>8</td>
<td>79*</td>
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<td>0</td>
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</tr>
<tr>
<td>12763</td>
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<td>Pf25</td>
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<td>8</td>
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<td>15</td>
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<tr>
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<td>F</td>
<td>naïve</td>
<td>204*</td>
<td>10</td>
<td>92*</td>
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<tr>
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<td>445*</td>
<td>10</td>
<td>106</td>
<td>12.6</td>
<td>0.23</td>
<td>19</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**TABLE 1**

*PMSP-1<sub>142</sub> = *P. falciparum* merozoite surface antigen-1, 2 mg IM administered on Weeks -8, -5, and -2 prior to the first FVO challenge.*

*PIAMA-1 = *P. falciparum* apical membrane antigen-1, 2 mg IM administered on Weeks -9, -6, and -3 prior to the first FVO challenge.*

*FVO1-7 = *P. falciparum* DNA vaccine, 500 μg IM on Weeks -8, -5, and -2 prior to the first FVO challenge.*

*PC = highest parasite count in parasitemia/μl blood x 10<sup>7</sup>; means for parasite counts are geometric means.*

*PP = pre-patent period in days; means for lengths of pre-patency are arithmetic means.*

*nc = not challenged; GeoMean = geometric mean; M = male; F = female.*

*1 treated with three doses of mefloquine daily for three days (20 mg/kg).*

*2 treated with a single dose of mefloquine (20 mg/kg).*
after the sixth and seventh FVO challenges. Six of eight of the monkeys became parasitemic after the CAMP challenge, as did all three naive monkeys challenged simultaneously with the CAMP strain (Table 1). The difference between the 100% protected from detectable parasitemia in the seventh FVO challenge and the 25% protected in the CAMP challenge was significant ($P = 0.007$, Fisher exact test two-tailed).

**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 $1.2 \times 10^5$ 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 $1.8 \times 10^6$ parasites/μl (geometric mean = 106,621 parasites/μl). All monkeys that became parasitic during FVO infections 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 parasitic 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 parasitic 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 $7.7 \times 10^4$ parasites/μl.

**Hematocrit.** After the CAMP challenge, mean hematocrits 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 hematocrit 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 melquine 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
day. Were the difference 1.2 days, we would have had a 0.5 probability of detecting that true difference. In the second FVO challenge, we had a 0.38 probability of detecting a true difference of 1.75 days. Were the difference 2.1 days, we would have had a 0.5 probability of detecting that true difference. In the CAMP challenge, we had a 0.15 probability of detecting a true difference of 2 days. Were the difference 4.5 days, we would have had a 0.5 probability of detecting that true difference. There was no association between immunization status and frequency of treatment in any of the infections (Chi square, $P = non-calculable$, $P = 0.99$, $P = non-calculable$, respectively).

**DISCUSSION**

*Atous* 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 *Atous* monkeys. These studies, performed in other species of *Atous*, also showed that previous infection provided protection from subsequent challenge with the same strain. There was also demonstrations of varying degrees of protection to heterologous challenge, but a variety of differences between those studies and work reported here include different *Atous* species, different *P. falciparum* strains, and the use of spleenectomized 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 *Atous*, 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 had been reported for both FVO and CAMP. In region II, which includes the glycoprotein A binding domain, of erythrocyte binding protein 175 (EBA-175), four of 616 (0.65%) amino acids were non-identical (accession X52524; accession AAU7390). The reported sequences for FVO and CAMP AMA-1 were 3,37% non-identical (21 of 622 residues) (accession M58545, Aguiar 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 10 million *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 10 million *Atous* 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, $P = 0.013$, one-way ANOVA). In addition, the frequency of treatment for parasitemia or clinical deterioration decreased from 100% during the first challenge to 63% during the second challenge, then to 0% in subsequent challenges (Table 1). Others have reported a similar ability to protect both *Atous* 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.

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**Table 2**

Hematocrits in the CAMP-challenged immune *Aotus* ($n = 8$) and the naïve control *Aotus* ($n = 3$). Days cited are days post-CAMP challenge. The right column (%) drop shows the percent the hematocrit decreased from Day 10 to its lowest level on any subsequent day.

<table>
<thead>
<tr>
<th>Monkey no</th>
<th>Maximum parasitemia</th>
<th>Day*</th>
<th>Parasitemia</th>
<th>Hematocrit (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12749</td>
<td>0</td>
<td>–</td>
<td>Day 10</td>
<td>Day 14</td>
</tr>
<tr>
<td>12759</td>
<td>2520</td>
<td>14</td>
<td>53</td>
<td>52</td>
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<td>9</td>
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<td>12943</td>
<td>767250</td>
<td>13</td>
<td>55</td>
<td>46</td>
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</tbody>
</table>

* day upon which the maximum parasite density occurred.
1 lowest hematocrit recorded for that animal.
2 treated, meloquine 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.

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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 hematocrit (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 they developed parasite densities like those seen in monkeys 12765 and 12763 (10,000 parasites/µl or greater), the travelers would become ill and a 50% decrease in their hematocrits, even if not to life-threatening levels, would certainly have a clinical impact.

The fact that the pre-patent period after the heterologous CAMP challenge was similar to the pre-patent period following the first FVO infection (not significantly different, $P = 0.6$, t-test) suggests to us that immediately after the CAMP infection, the anti-FVO immune responses had only a modest effect, if any, on controlling the infection. However, the antigens presented at the beginning of the CAMP infection must have led to rapid boosting of cross-reacting anti-CAMP immune responses primed by repeated FVO infections, and these immune responses controlled the parasitemia.

The rapid reduction in hematocrit in some of the FVO-immune animals challenged with CAMP is disturbing. The dramatic decrease in hematocrit in monkeys 12765, 12763, and 12756 (Table 2) cannot be ascribed to destruction of infected erythrocytes, since peak parasitemias were only 10,920 parasites/µl. If one assumes $3.3 \times 10^9$ erythrocytes/µl blood, then this level of parasitemia would translate into only 0.33% of erythrocytes being infected. Destruction of these cells cannot account for a decrease in hematocrit of over 50%. Ineffective erythrocytosis cannot be invoked for this drop which occurred over 7 to 11 days. We believe that only hemolysis of non-infected erythrocytes can explain the rapid decrease in hematocrit that we observed, and we are currently evaluating the anemia from this perspective.

Although some of the monkeys received DNA immunizations prior to the start of this study, we were unable to document any effect associated with immunization status. Although the two naive animals (12739 and 12749) were the only animals that did not become parasitemic upon CAMP challenge, it should be remembered that the two others immunized with Pf525 are, for the purposes of this study, also naive because Pf525 is not expressed by asexual parasites.

These two animals (12765 and 12763) had the densest CAMP parasitemias. Attempting to draw conclusions about 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 *Plasmodium 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.

Acknowledgments: The authors thank Dr. William O. Rogers and Dr. Richard Hedstrom for comparing the FVO and CAMP antigen sequences available through NCBI. The authors also thank Ms. Gloria Cisneros for supervision of animal husbandry at the Gorgas Memorial Institute Research Facility, Mr. Lionel Martinez and Mr. William Otero, also of the Gorgas Memorial Institute, for performing the microscopic analysis of blood films generated in this study, and Ms. M. Brewer for secretarial support.

Disclaimer: The opinions and assertions herein are the private ones of the authors and are not to be construed as official or as reflecting the views of the U.S. Navy or the U.S. Department of Defense.

Financial support: This work was supported by the Naval Medical Research and Development Command, work units 611193.1300110-1901, 61277A.870.00110, 1431 and 61277A.870.00110, and BFX.1342 and 61277A.870.00110, and U.S. Army Contract DAMD17-91-C-1072.


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