TESTING THE EFFICACY OF A RECOMBINANT MEROZOITE SURFACE PROTEIN (MSP-1\textsubscript{19}) OF *PLASMODIUM VIVAX* IN SAIMIRI BOLIVIENSIS MONKEYS

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Abstract. *Saimiri boliviensis* monkeys were immunized with the yeast-expressed recombinant protein yP\textsubscript{2}P\textsubscript{30}Pv200\textsubscript{19}. The antigen consisted of the C-terminus (amino acid Asn\textsubscript{1622}-Ser\textsubscript{1729}) of the merozoite surface protein 1 of the *Plasmodium vivax* Salvador I strain. Two universal T helper cell epitopes (P\textsubscript{2} and P\textsubscript{30}) of tetanus toxin and six histidine residues for purification purposes were attached to the N- and C-termini, respectively. Four groups of five monkeys were given three immunizations at four-week intervals with either 250 µg of yP\textsubscript{2}P\textsubscript{30}Pv200\textsubscript{19} formulated with nonionic block copolymer P1005, 250 µg of antigen adsorbed to alum, 250 µg of antigen in phosphate-buffered saline (PBS), or PBS alone. Five weeks after the last immunization, each animal was inoculated with 100,000 parasitized erythrocytes of the Salvador I strain of *P. vivax*. Animals were splenectomized one week after challenge to increase parasite densities; after seven weeks of infection, animals were treated. Eighteen weeks later, the animals were rechallenged with the homologous parasite. Following the first challenge, three monkeys immunized with the antigen with P1005 were protected; no animals were protected from rechallenge. One monkey immunized with yP\textsubscript{2}P\textsubscript{30}Pv200\textsubscript{19} with alum was protected; no protection was seen after rechallenge. Two monkeys immunized with antigen alone were protected; none were protected from rechallenge. One control animal had a low parasite count following primary infection; none were protected against rechallenge. Adverse reactions were only observed with animals receiving P1005. It is proposed that splenectomy of the monkeys prevented adequate assessment of the efficacy of this antigen. Identification of a monkey host that supports high density parasitemia without splenectomy appears needed before further testing of blood-stage vaccines against *P. vivax*.

Procedures are being developed and evaluated for the testing of different synthetic peptide and recombinant protein vaccines in animals susceptible to infection with different species of *Plasmodium*. A model system has been developed for the testing of vaccines directed against the sporozoites of *Plasmodium vivax* using the Salvador I strain of the parasite and the nonhuman primate model of *Saimiri boliviensis boliviensis*. In previous trials, immunized animals were challenged with 10,000 or more sporozoites dissected from the salivary glands of infected mosquitoes, and the animals were splenectomized one week after challenge to increase parasitemia. Infection rate was highly predictable, but prepatent periods varied markedly.

Our present study was designed to determine if this model system could be used to test the efficacy of a recombinant vaccine directed against the merozoite surface protein (MSP-1\textsubscript{19}) of *P. vivax*. To increase uniformity of parasitemia, all animals were challenged by the intravenous inoculation of parasitized erythrocytes. All animals were splenectomized seven or eight days after challenge. Included in the trial were two adjuvants, a nonionic block copolymer P1005, previously shown to increase antibody responses, and alum, the standard human-usable adjuvant.

Reported here are the results of this trial to assess efficacy by daily monitoring of peripheral parasitemia. After termination of the primary infection, animals were rechallenged with the homologous parasite to determine if there was persistence of immunity in these splenectomized hosts.

MATERIALS AND METHODS

**Vaccine.** The antigen was a yeast-expressed recombinant protein yP\textsubscript{2}P\textsubscript{30}Pv200\textsubscript{19}. It consists of the C-terminus (amino acid Asn\textsubscript{1622}-Ser\textsubscript{1729}) of the merozoite surface protein 1 of the *P. vivax* Salvador I strain. In addition, two universal T helper cell epitopes (P\textsubscript{2} and P\textsubscript{30}) of tetanus toxin and six histidine residues for purification purposes were attached to the N- and C-termini, respectively. The N-terminal sequence determined by Edman degradation showed that the prominent species starts at Leu\textsubscript{1639}, which is approximately where Pv200 is predicted to be cleaved by the parasite. The expression, purification, and characterization of the antigen are described in detail elsewhere.

**Animals.** Twenty (12 females and 8 males) *S. boliviensis boliviensis* monkeys of Bolivian origin were used in the study. All animals were quarantined upon arrival at the facility for at least one month, weighed, and tested by skin test for tuberculosis. Parasitologic and serologic examination indicated that the animals were free of plasmodial infection. Protocols were reviewed and approved by the Centers for Disease Control and Prevention Institutional Animal Care and Use Committee, in accordance with procedures described in the U. S. Public Health Service Policy, 1986. For six weeks before immunization and continuing throughout the trial, daily observations of the animals attitude, appetite, stool, and condition were recorded. Monkeys were weighed weekly. All were treated as medical conditions arose by the attending veterinarian. All observations, parasite counts, and the results of laboratory tests were recorded on a daily basis and entered into a computerized database.

Animals were housed singly or doubly to avoid injuries caused by fighting with cagemates. Space recommendations for laboratory animals were followed as set forth in the *Guide for the Care and Use of Laboratory Animals, NIH*. All monkeys were fed a diet that has been shown to provide adequate nutrition and calories in captive *Saimiri* used in malaria-related research. Feed was free of contaminants and freshly prepared. Animals were handled as little as possible.
They were administered 0.1 ml of ketamine hydrochloride intramuscularly for sedation when given a physical examination, had blood drawn, and were weighed. On days when only blood was drawn or the animals were weighed only, anesthesia was not given. All procedures involving the animals were under the direction of the resident clinical veterinarian.

Recorded observations on local and/or systemic reactions (e.g., lymphadenopathy, cellulitis, abscesses, necrotizing lesions, arthritis, anorexia, and weight loss) to the candidate vaccine were made minimally once a week at the time of blood collection. In the event of a reaction, additional observations were made daily, and supportive treatment was instituted.

**Immunization schedule.** Animals were assigned in four groups of five using a table of random numbers taking into account sex and weight. Animals were given three immunizations at four-week intervals (identified as weeks minus $-13$, $-9$, and $-5$ (Figure 1). Day 0 (week 0) was the day of the first challenge. Immunizations were given subcutaneously in four sites in the back of each animal. The injection sites were shaved to allow visual monitoring for any adverse reactions. Each of the animals in Group I received 250 $\mu$g of the $yP_{2}P_{30}P_{200}$ recombinant antigen formulated with non-ionic block copolymer P1005 in a water-in-oil emulsion each immunization. Group II animals received 250 $\mu$g of the recombinant antigen adsorbed to aluminum hydroxide at each injection. Group III animals were injected with 250 $\mu$g of the recombinant antigen in phosphate-buffered saline (PBS) each immunization. Group IV animals were immunized with PBS alone. The trial was conducted in a blind format. Persons responsible for the reading of blood smears for parasitemia determinations, conduct of serologic testing, collection of specimens, examination and provision of health care, performing the required surgical procedures, and for the statistical analysis of the data did not know the experimental group to which each animal had been assigned. The same person was responsible for administering immunizations.

**Challenge.** Five weeks after the last immunization (week 0), each animal was inoculated with 100,000 parasitized erythrocytes of the Salvador I strain of $P. \text{vivax}$ taken from a donor animal, diluted in RPMI 1640 medium, and injected intravenously into the femoral vein of each animal. For the rechallenge (week 25), each animal was injected with 100,000 erythrocytes parasitized with the same strain of $P. \text{vivax}$.

**Surgery.** Splenectomies were performed seven or eight days after the primary challenge under general anesthesia by a qualified veterinarian. All surgeries were performed in an Association for the Assessment and Accreditation of Laboratory Animal Care, International, Inc.—approved surgical suite appropriate for aseptic surgery as previously described.

**Parasite monitoring.** Beginning one day after challenge, thick and thin blood films were collected daily and stained with Giemsa by the method of Earle and Perez. Parasite counts were recorded per microliter of blood. Following the first parasitized erythrocytic challenge, blood films were made and examined from days 1 through 42 (weeks 0 through 7). Following the second challenge, blood films were examined from days 1 to 28 (weeks 25 through 29).

Forty-two days after the first challenge, all animals were treated orally with 20 mg of mefloquine, which cured their infections. Eighteen weeks after termination of the primary infections (week 25), 18 animals remaining in the trial were rechallenged.

**Hematology and serology.** Biweekly collections of $<10\%$ of each monkey’s estimated total blood volume (based on a total blood volume calculation of 40 ml of blood per kilogram of body weight) were made via venipuncture from the femoral vein. A complete blood count was done: 1) erythrocytes/$\mu$l; 2) leukocytes/$\mu$l; 3) hematocrit; 4) hemoglobin concentration; 5) platelets/$\mu$l; 6) mean corpuscular hemoglobin; 7) mean corpuscular volume; and 8) mean corpuscular hemoglobin concentration. The remaining blood

was centrifuged and the plasma stored frozen for serologic and blood chemistry studies. For lymphoproliferation assays, peripheral mononuclear cells were collected from the monkeys before the first immunization, two weeks after the second and third immunizations, and four weeks after the first challenge. The methods and results of the serologic and proliferation assays following immunization and primary challenge infection are presented elsewhere. Here, we report on the parasitologic and serologic results following rechallenge.

RESULTS

Before the first challenge, two monkeys, one from immunization Group II and one from control Group IV, died from causes unrelated to the immunization. The parasite data were examined for maximum parasite count and accumulated parasite counts over the periods following each challenge.

**Group I.** Five monkeys immunized with yP2P,30Pv20019 combined with block copolymer P1005 survived through the immunization, two challenges, and subsequent infection (Figures 2A and 3A). Following the first challenge (Table 1 and Figure 2A), three monkeys (SI-1024, SI-2026, and SI-2053) had maximum parasite counts of 10, 20, and 20/μl, respectively, and accumulated daily parasite counts over the 42 days following challenge of 25, 30, and 120 parasites/μl, respectively. The other two animals (SI-2119 and SI-2063) had maximum parasite counts of 24,170 and 40,140/μl and accumulated daily parasite counts of 93,399 and 291,391, respectively. Prepatent periods ranged from 7 to 15 days with a mean of 11.4 days.

On the day of rechallenge, an ELISA indicated high titers of antibody to the yP2P,30Pv20019 antigen were present in sera from all five animals (Table 1). Following the rechallenge, (Table 1 and Figure 3A), the five monkeys (SI-1024, SI-2125, SI-2053, SI-2119, and SI-2063) had maximum parasite counts of 16,740, 29,700, 18,544, 14,362, and 6,120/μl, and accumulated daily parasite counts were 68,067, 70,560, 127,598, 80,494, and 46,717, respectively. Prepatent periods ranged from three to eight days with a mean of 6.4 days. Protection, which was apparent during the primary challenge following immunization, was not evident following rechallenge 29 weeks after the last immunization. The total accumulated daily parasite count for the five animals following both challenges was 778,401/μl (155,680/animal). Twenty-eight days after infection, the ELISA titers were essentially the same as at the day of inoculation, ranging from 1:51,200 to 1:204,800.

**Group II.** Four monkeys immunized with yP2P,30Pv20019
Figure 3. Parasitemia curves for *Saimiri boliviensis boliviensis* monkeys following secondary challenge with 100,000 parasitized erythrocytes of the Salvador I strain of *Plasmodium vivax*. **A**, five monkeys immunized with yP$_2$P$_3$0 Pv200$_19$ with nonionic block copolymer P1005. **B**, four monkeys immunized with yP$_2$P$_3$0 Pv200$_19$ with alum. **C**, five monkeys immunized with yP$_2$P$_3$0 Pv200$_19$ in phosphate-buffered saline. **D**, four control monkeys.

Table 1
Parasitologic and serologic responses in *Saimiri boliviensis boliviensis* monkeys immunized with a fused polypeptide yP$_2$P$_3$0 Pv200$_19$ corresponding to the merozoite protein 1 of *Plasmodium vivax*.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Antigen + adjuvant</th>
<th>Prepatent period</th>
<th>Max. para.</th>
<th>Accum. para.</th>
<th>ELISA day 0</th>
<th>Prepatent period</th>
<th>Max. para.</th>
<th>Accum. para.</th>
<th>ELISA day 28</th>
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<tr>
<td>SI-1024</td>
<td>PV200 + P1005</td>
<td>15 days</td>
<td>10</td>
<td>16</td>
<td>25</td>
<td>51,200</td>
<td>3 days</td>
<td>16,740</td>
<td>15</td>
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<tr>
<td>SI-2126</td>
<td>PV200 + P1005</td>
<td>13 days</td>
<td>20</td>
<td>13</td>
<td>30</td>
<td>204,800</td>
<td>8 days</td>
<td>29,700</td>
<td>28</td>
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<tr>
<td>SI-2053</td>
<td>PV200 + P1005</td>
<td>14 days</td>
<td>20</td>
<td>22</td>
<td>120</td>
<td>204,800</td>
<td>7 days</td>
<td>18,544</td>
<td>26</td>
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<tr>
<td>SI-2119</td>
<td>PV200 + P1005</td>
<td>15 days</td>
<td>31,420</td>
<td>35</td>
<td>93,399</td>
<td>102,400</td>
<td>8 days</td>
<td>14,362</td>
<td>19</td>
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<tr>
<td>SI-2063</td>
<td>PV200 + P1005</td>
<td>7 days</td>
<td>40,140</td>
<td>23</td>
<td>291,391</td>
<td>204,800</td>
<td>6 days</td>
<td>6,120</td>
<td>15</td>
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<tr>
<td>SI-1021</td>
<td>PV200 + Alum</td>
<td>12 days</td>
<td>60</td>
<td>21</td>
<td>321</td>
<td>3,200</td>
<td>9 days</td>
<td>64,000</td>
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<td>SI-2083</td>
<td>PV200 + Alum</td>
<td>8 days</td>
<td>2,727</td>
<td>27</td>
<td>27,925</td>
<td>6,400</td>
<td>4 days</td>
<td>168,000</td>
<td>26</td>
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<tr>
<td>SI-2016</td>
<td>PV200 + Alum</td>
<td>10 days</td>
<td>7,909</td>
<td>30</td>
<td>37,789</td>
<td>102,400</td>
<td>9 days</td>
<td>10,530</td>
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<tr>
<td>SI-2021</td>
<td>PV200 + Alum</td>
<td>9 days</td>
<td>41,580</td>
<td>40</td>
<td>485,947</td>
<td>51,200</td>
<td>12 days</td>
<td>64,000</td>
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<td>242</td>
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<td>SI-2143</td>
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<td>32</td>
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<td>SI-2109</td>
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<td>41,580</td>
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<td>PV200</td>
<td>7 days</td>
<td>35,087</td>
<td>26</td>
<td>460,524</td>
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<td>SI-2105</td>
<td>Control</td>
<td>14 days</td>
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<td>100</td>
<td>11 days</td>
<td>80,000</td>
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<td>SI-2026</td>
<td>Control</td>
<td>8 days</td>
<td>8,272</td>
<td>26</td>
<td>68,536</td>
<td>400</td>
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<td>SI-2077</td>
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<td>8 days</td>
<td>16,020</td>
<td>42</td>
<td>54,587</td>
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<td>SI-0793</td>
<td>Control</td>
<td>11 days</td>
<td>25,200</td>
<td>42</td>
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<td>1,600</td>
<td>5 days</td>
<td>84,000</td>
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*Max. para.* = maximum parasitemia; *Accum. para.* = accumulated parasitemia.
combined with alum survived (Table 1 and Figures 2B and 3B). One monkey, SI-1021, following the primary challenge had a maximum parasite count of 60/μl, and an accumulated daily parasite count of 321/μl. The other three animals (SI-2083, SI-216, and SI-2021) had maximum parasite counts of 2,727, 7,909, and 41,580, and accumulated daily parasite counts of 27,925, 37,789, and 485,947/μl, respectively. Prepatent periods ranged from 8 to 12 days with a mean of 9.75 days. Following rechallenge (Figure 3B), monkey SI-1021, the animal protected following the primary challenge, had a maximum parasite count of 64,000/μl and an accumulated daily parasite count of 429,497/μl. The other three animals (SI-2083, SI-216, and SI-2021), had maximum parasite counts of 168,600, 10,543, and 18,540/μl, respectively, and accumulated daily parasite counts of 883,894, 18,878, and 107,551/μl, respectively. Prepatent periods ranged from 4 to 12 days with a mean of 8.5 days. The accumulated daily parasite count for the four animals following both challenges was 1,991,802/μl. Prepatent periods ranged from 8 to 12 days with a mean of 8.0 days. The accumulated daily parasite count was 100/μl. The accumulated daily parasite count for the four animals following both challenges was 1,626,855/μl (406,714/animal). At the time of rechallenge, the ELISA titers ranged from 1:100 to 1:1,600; 28 days later, titers ranged from 1:800 to 1:204,800.

**Adverse reactions.** No significant adverse response occurred at the immunization sites in the animals receiving the antigen with alum (Group II), the antigen alone (Group III), or in the control animals. However, all five animals receiving yP 2 P 30 Pv200 19 with the block copolymer P1005 (Group I) had some degree of adverse reaction at one or more of the immunization sites following the third immunization. Of the five monkeys in Group I (Table 2), the responses ranged from 1+ (erythema or induration), 2+ (erythema and induration), 3+ (erythema, induration, and edema), to 4+ (ulceration).

**DISCUSSION**

Parasitemia with the Salvador I strain of *P. vivax* in non-splenectomized *S. boliviensis boliviensis* monkeys has been difficult to predict. Therefore, in our previous vaccine trials with vaccines directed against sporozoites, animals have been splenectomized 6–8 days after sporozoite injection, after the predicted period of protective activity against the sporozoites and/or exoerythrocytic stages is completed. At that time, very few or no preerythrocytic stages are present. Splenectomy allows for the parasitemia to increase to readily detectable levels for assessment of vaccine efficacy. In the use of this model for assessment of blood-stage vaccines, splenectomy was delayed until six weeks after completion of immunization and one week after the primary challenge. Erythrocytic stages are already present in the circulation. This schedule was expected to allow for the development of protective immunity induced by a vaccine, yet increase the uniformity of subsequent parasitemia in both the immunized and the control animals. Such a scheme has been used by us in the assessment of blood-stage vaccines in *Aotus* monkeys being challenged with less virulent strains of *P. falciparum* (unpublished data).

An examination of the serologic response against the yP 2 P 30 Pv200 19 antigen indicated geometric mean reciprocal ELISA titers (GMRTs) for immunization groups I, II, and III of 135,100, 18,100, and 1,600, respectively; control animals (Group IV) had a mean titer of 475. Twenty-eight days after rechallenge, the GMRTs for these four groups of monkeys were 117,600, 60,900, 102,400, and 43,050, respectively. Group I had the highest GMRT on the day of rechallenge and maximum parasite count or accumulated parasitemia. Of the five monkeys in Group I (Table 2), the responses ranged from 1+ (erythema or induration), 2+ (erythema and induration), 3+ (erythema, induration, and edema), to 4+ (ulceration).

In the study reported here, protection was suggested in the animals immunized with yP 2 P 30 Pv200 19 combined with non-ionic block copolymer P1005. However, death of one of the control monkeys, and the failure of control monkey SI-2105 to develop high-density parasitemia, in spite of splenectomy, made significant analysis impossible. Nonetheless, an examination of the accumulated parasite counts following the two challenges suggested that there was a beneficial effect...
of immunization with the antigen combined with P1005. The mean accumulated parasite counts for the five monkeys immunized with yPfvPv200₁₀ with P1005 was 155,680 per animal; for the four monkeys immunized with yPfvPv200₁₀ with alum, the accumulated parasitemia per animal was 497,950; for the five monkeys immunized with yPfvPv200₁₀ only, it was 375,016 per animal; and for the four control monkeys it was 406,714 per animal.

The Salvador I strain of P. vivax in S. boliviensis boliviensis is the best combination of parasite and monkey host we have found suitable for testing anti-sporozoite vaccines against this parasite. To adequately assess efficacy, splenectomy after sporozoite challenge was considered necessary. For our present study, we attempted to use the same model system for the testing of blood-stage vaccines; the results were only marginally successful. This may have been due more to the unsuitability of the model than to any fault with the vaccine preparations. Splenectomy has been used to markedly increase the growth of erythrocytic infections with P. vivax in Saimiri and Aotus monkeys. It appears that splenectomy may have had such an effect on both the immunized and nonimmunized animals in this trial. In addition, splenectomy may have greatly reduced the positive effect that immunization may have generated. The lack of clear-cut protection suggests that blood-stage vaccine trials could better be conducted in intact rather than splenectomized hosts. In our immunization and challenge trials with candidate blood-stage vaccines against P. falciparum, we have used intact rather than splenectomized Aotus monkeys. A similar procedure should be used for the assessment of blood-stage vaccines against P. vivax.

We believe that a strain of P. vivax capable of inducing predictable high-density parasitemia in intact animals is needed before further evaluation of candidate blood-stage vaccines against P. vivax in New World monkeys. The Salvador I strain of P. vivax in S. boliviensis boliviensis appears to be unsatisfactory for these blood-stage vaccine trials. Although Aotus monkeys are usually less susceptible to infection via sporozoite inoculation, many strains of this parasite have been adapted to develop in Aotus monkeys. Further studies to assess the efficacy of yPfvPv200₁₀ and other blood-stage candidate vaccines may better be conducted in Aotus monkeys using a strain of P. vivax shown to induce predictably high-density parasitemia in intact animals. Attempts will be made to establish such a model using strains of the parasite already adapted to monkeys. Once such a model is characterized, further trials of candidate blood-stage vaccines against P. vivax should be more meaningful.

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Table 2

<table>
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<th>SI-2053</th>
<th>SI-2063</th>
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<tr>
<td>Immunization II</td>
<td>35 ± ± ± ±</td>
<td>35 ± ± ± ±</td>
<td>50 – – – –</td>
<td>56 + + + +</td>
<td>63 ± ± ± ±</td>
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<tr>
<td>Immunization III</td>
<td>70 2+ 2+ 2+ 2+</td>
<td>77 ± ± ± ±</td>
<td>85 – – – –</td>
<td>91 – – – –</td>
<td>105 – – – –</td>
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<td>Challenge</td>
<td>98 – – – –</td>
<td>113 2+ – – 3+ 4+ – –</td>
<td>119 – – – –</td>
<td>127 – – – –</td>
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<td>105 – – – –</td>
<td>113 2+ – – 3+ 4+ – –</td>
<td>119 – – – –</td>
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<tr>
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<td>156 – – – –</td>
<td>162 – – – –</td>
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</table>

*p UR = upper right site; UL = upper left site; LR = lower right site; LL = lower left site. – = no reaction; ± = slight erythema; + = erythema or induration; 2+ = erythema and induration; 3+ = erythema, induration, and edema; 4+ = ulceration.

P. VIVAX VACCINE TRIAL IN MONKEYS

355
REFERENCES


