SALVADOR II STRAIN OF *PLASMODIUM VIVAX* IN *AOTUS* MONKEYS AND MOSQUITOES FOR TRANSMISSION-BLOCKING VACCINE TRIALS

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Abstract. Infections with the Salvador II strain of *Plasmodium vivax* in *Aotus lemurinus griseimembra* monkeys were fed upon by *Anopheles freeborni* mosquitoes. Periods of mosquito infectivity were determined to establish a model system for the testing of transmission-blocking vaccines. The highest levels of mosquito infection were associated with the ascending asexual parasitemia after reaching 1,000/μL and before the peak asexual parasite count. Sporozoite-induced infections were more infectious than were trophozoite-induced infections. Secondary episodes of parasitemia were also infectious, indicating the lack of development of naturally developing transmission-blocking immunity to this strain of *P. vivax* in splenectomized *Aotus* monkeys following single infections.

Previously, we have reported on the infection of different New World monkeys as models for specific strains of human malaria parasites for biologic and immunologic studies. The Vietnam Oak Knoll strain of *Plasmodium falciparum* in *Aotus nancymai* was suitable for the conduct of reproducible trials against blood-stage vaccines.1–5 The Santa Lucia strain of *P. falciparum* was suitable for transmission-blocking and anti-sporozoite vaccines in *Aotus lemurinus griseimembra* and *A. vociferans* monkeys.6, 7 The Salvador I strain of *P. vivax* was suitable for vaccine trials with sporozoite vaccines in splenectomized *Saimiri boliviensis* monkeys.8–13 Reported here are the results of observations on the Salvador II strain of *P. vivax*, indicating the usefulness of infections with this parasite in *A. lemurinus griseimembra* monkeys for immunologic and vaccine studies with transmission-blocking vaccines.

The characteristics needed for an animal model to test transmission-blocking vaccines are 1) frequent and sustained production of infective gametocytes, and 2) mosquitoes that are readily susceptible to infection. A predictable pattern of infection allows for the design of specific time frames for the assessment of efficacy.

The Salvador II strain of *P. vivax* has been studied extensively since its isolation into *Aotus* monkeys and is readily infectious to different species and strains of *Anopheles.*14–24 Reported here are the results of our studies with the Salvador II strain of *P. vivax* in *A. lemurinus griseimembra* monkeys and *An. freeborni* mosquitoes; this combination provides the desired characteristics for immunologic and transmission-blocking vaccine trials.

MATERIALS AND METHODS

The Salvador II strain of *P. vivax* was originally isolated into an *A. lemurinus griseimembra* monkey from a natural infection in the region of Las Guarumas, in the state of La Paz, El Salvador.14 The parasite has been maintained by serial passage or stored frozen over liquid N₂.

The *A. lemurinus griseimembra* monkeys were obtained commercially or were laboratory born. These animals (karyotypes K-II, K-III or K-IV), originated from Colombia. Animals were infected by the intravenous inoculation of parasitized blood either fresh or from aliquots stored over liquid N₂, or by the injection of sporozoites. Sporozoite-induced infections were induced either via the bites of infected mosquitoes or the intravenous and/or intrahepatic inoculation of sporozoites dissected from the salivary glands of infected mosquitoes. All animals were splenectomized under aseptic conditions before infection. Animals were housed in an American Association for Accreditation of Laboratory Animal Care–approved facility under the supervision of a clinical veterinarian. Studies were reviewed and approved by the Institutional Animal Care and Use Committee according to Public Health Service Policy, 1986.

During the infection, thick and thin blood films were made daily according to the technique of Earle and Perez,25 stained with Giemsa stain, and the parasite counts were recorded per microliter of blood.

The *An. freeborni* mosquitoes were originally from Marysville, California, and had been maintained in the laboratory since 1944. The procedures used for feeding, handling, and dissection of the mosquitoes have been described previously.26 Extrinsic incubation was at 25 ± 1°C and ≥ 60% relative humidity. Mosquitoes were examined for the presence of oocysts from 5 to 10 days after feeding on the monkeys; salivary glands were examined beginning 12 days after infection.

For sporozoite-induced transmission, infected mosquitoes were allowed to feed on tranquilized monkeys, or sporozoites were dissected from the salivary glands into serum saline, quantitated using a Neubauer cell counting chamber, and injected intravenously or intrahepatically into the monkeys. For blood-induced infections, parasitized erythrocytes, either freshly collected from donor animals or from blood that has been stored frozen, were injected intravenously into the femoral vein.

RESULTS

Eighty-two splenectomized monkeys were infected either by the inoculation of parasitized erythrocytes (42 animals) or sporozoites (40 animals). The objective of these studies was to determine those times during the course of parasitemia when mosquito infection could be obtained for the evaluation of transmission-blocking vaccines. Thus, a retrospective analysis was made based on several different bases: 1) the infection of *An. freeborni* mosquitoes during the primary attack in relation to the peak asexual parasite count,
2) the infection of An. freeborni mosquitoes in relation to the primary increase in the parasite count, 3) the infection of An. freeborni mosquitoes in relation to the peak asexual parasite count during secondary courses of parasitemia, and 4) a comparison between sporozoite-induced and blood-induced infection. Numbers were based on oocyst counts. The analysis was based on the course of asexual parasitemia because gametocytes were rarely observed in the blood films of monkeys infected with *P. vivax*.

Mosquito infection in relation to peak asexual parasite count. Mosquito infection was determined during the primary attack for the 12 days before the peak asexual parasite count and continuing through 11 days following the peak (Figure 1A). Of 1,114 lots of An. freeborni fed, dissected, and examined, 624 (56.0%) were infected. Greater than 50% of the lots were infected on days -12 through the first day following the peak asexual parasite count; the rate then diminished through day 11. Of the 23,283 mosquitoes examined, 5,263 had one or more oocysts on the midgut. The average percentage of the mosquitoes infected was determined for each day in relation to the peak parasite count (Figure 2A). Greater than 25% of the individual mosquitoes in each lot were infected through the peak day of asexual parasite count; thereafter, oocyst infection decreased markedly. It was apparent that the factors responsible for the decrease in asexual parasitemia coincided with the reduction in gametocyte infectivity.

A similar examination was made of the results of mosquito feeding on secondary peaks in the asexual parasite count. Of 1,335 lots of An. freeborni fed and examined during this period, 832 (62.32%) of the lots were positive (Figure 1B). Greater than 50% of the lots were positive as late as seven days following the peak asexual parasite count. Of the 27,992 mosquitoes examined, 6,216 had at least one oocyst on the midgut. Greater than 25% of the mosquitoes within the individual lots were infected beginning 10 days before the peak asexual parasite count and extended through two days following the peak (Figure 2B).

An examination was made of those periods when the intensity of oocyst infection was greatest for both the primary infections and secondary peak periods (Figure 3A). During the primary attack, lots of mosquitoes with oocyst counts averaging greater than 10 per gut occurred as early as 12 days before the peak and extended through day 6 following the peak; highest incidence of such highly infected lots of mosquitoes occurred between five days before the peak through one day after the peak parasite count. For the secondary infections (Figure 3B), lots of mosquitoes with high density oocyst counts were distributed around the peak asexual parasite count. For the primary infections, there were 624...
positive lots of mosquitoes of which 334 had average oocyst densities between 1 and 10 per gut (53.5%); 127 had average oocyst densities of greater than 10 per gut (20.4%). For the mosquitoes fed during secondary peak parasitemia, 341 of the 832 positive lots had oocyst densities between 1 and 10 per gut (41.0%); 92 had average oocyst densities of greater than 10 per gut (11.1%).

**Mosquito infection in relation to an increase in asexual parasite count.** A further analysis was made of the data based on the ascending parasite count during the primary attack in sporozoite-induced versus trophozoite-induced infections. Mosquito infection was determined during the primary attack for sporozoite-induced and trophozoite-induced infections beginning three days before the asexual parasite count reached 1,000/μl and continuing for the following 20 days. A total of 1,031 lots of *A. freeborni* were fed, dissected, and examined; 370 of 550 lots fed on sporozoite-induced infections were positive (67.3%) versus 215 of 481 lots fed on trophozoite-induced infections (56.7%). Greater than 50% of the lots fed on sporozoite-induced infections were positive on days −3 through day 12 (Figure 4A), whereas percentages of 50% or greater were obtained on days −2 and days 1 through 5 for trophozoite-induced infections.

Of the 12,043 mosquitoes examined that fed on sporozoite-induced infections, 3,771 had one or more oocysts on the midgut. The average percentage of the mosquitoes infected was determined for each day in relation to the ascending parasite count (Figure 5A). Greater than 25% of the individual mosquitoes in each lot were infected from days −3 through day 11. Of the 9,700 mosquitoes that fed on trophozoite-induced infections, 1,248 had at least one oocyst on the midgut. Greater than 25% of the mosquitoes within the individual lots were infected beginning at day −3 and continuing through day 4; day 9 also had an average of greater than 25% of the mosquitoes infected.

An examination of those periods when the intensity of oocyst infection was greatest for both the sporozoite-induced and trophozoite-induced infections during the primary attack (Figure 6), indicated that the highest oocyst densities occurred between day 2 and day 9 after the asexual parasite count reached 1,000/μl.

**DISCUSSION**

The Salvador II strain of *P. vivax* in splenectomized *A. lemurinus griseimembra* monkeys was shown to be infective to *A. freeborni* mosquitoes in relation to the peak asexual parasite count as well as the ascending parasite count during the primary attack. *Anopheles freeborni* were more readily infected by feeding on sporozoite-induced than trophozoite-induced infections. Because the time of peak parasitemia is often difficult to predict, early commencement of mosquito feeding before the parasite count exceeded 1,000/μl would appear to be required for evaluation of the efficacy of trans-
mission-blocking vaccines. Although sporozoite-induced infections obviously better supported the development of infective gametocytes, trophozoite-induced infections also could be used for vaccine efficacy evaluation.

We propose that such vaccine assessment could be conducted in several ways. The primary method would be to immunize the monkeys, infect them with the Salvador II strain of *Plasmodium vivax*, and feed *An. freeborni* mosquitoes daily beginning as soon as the parasite count exceeds several 100/µl during the ascending primary parasitemia and continuing until the peak parasite count is attained. Unfortunately, non-splenectomized *Aotus* monkeys rarely support the development of infective gametocytes. Therefore, splenectomy before initiation of the study or at some time after immunization would be needed. Alternatively, intact animals would be immunized and serum samples collected at different time periods before, during, and following completion of the immunization schedule. This would allow the use of other species of primates that do not support the development of infective *P. vivax* gametocytes for immunization trials. Blood from nonimmunized animals infected by injection of sporozoites or trophozoites would be collected during the early ascending phase of the infection, mixed with sera from immunized animals, and fed to mosquitoes through artificial membranes. In this case, blood from one infected animal could be mixed with sera from several immunized animals, or with sera collected from an individual animal at different time periods, to assess the efficacy of immune sera. This procedure has been used with culture-produced gametocytes of *P. falciparum* to assess transmission-blocking immunity. However, since culture-produced *P. vivax* has not been shown to support the development of infective gametocytes, a patent infection is required to supply the needed infective gametocytes.

The results of feedings on secondary peak parasitemia presents information on the natural formation of transmission-blocking immunity. The results indicate that each episode of infection followed a similar pattern in the production of infective gametocytes. This suggests that the reduction in mosquito infectivity during the primary attack was due to a reduction in the production of infective gametocytes rather than the development of transmission-blocking immunity. The secondary increase in asexual parasite count is considered to be the presentation of a new population of asexual parasites sufficiently variant from the first that it is not controlled by the immune mechanisms brought to play against the primary parasite population. It would appear that transmission-blocking immunity in *P. vivax* is either very short lasting (or not at all), or the new variant population is able to overcome most of the transmission-blocking immunity that might have developed during the primary attack. The

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**Figure 4.** Percentage of lots of *Anopheles freeborni* infected with the Salvador II strain of *Plasmodium vivax* following feeding on sporozoite-induced (A) and trophozoite-induced (B) primary infections in splenectomized *Aotus lemurinus griseimembra* monkeys as related to the increase in parasite count. 1,000/µl. Day 1 = first day when the parasite count was > 1,000/µl.

**Figure 5.** Mean percentage of *Anopheles freeborni* mosquitoes infected with the Salvador II strain of *Plasmodium vivax* following feeding on sporozoite-induced (A) and trophozoite-induced (B) infections in splenectomized *Aotus lemurinus griseimembra* monkeys as related to the increase in parasite count. 1,000/µl.
results suggest that immune responses to exposed antigens of *P. vivax* present in the primate host may have minimal transmission-blocking activity. This contrasts with our previous observations with *P. falciparum* in which gametocytes produced during recrudescence in *A. lemurinus griseimembra* monkeys were seldom infective. However, when the host’s plasma was replaced with normal monkey plasma, gametocytes from the host were shown to be highly infectious. The current concept is that immunization with antigens associated with the stages within the mosquito may be of major importance for transmission-blocking activity. This may be most applicable for *P. vivax* vaccine development.

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