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SUSCEPTIBILITY OF PANAMANIAN *AOTUS LEMURINUS LEMURINUS* TO SPOROZOITE-INDUCED *PLASMODIUM FALCIPARUM* (SANTA LUCIA) INFECTION

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Abstract. *Aotus* monkeys are good models for erythrocyte-induced *Plasmodium falciparum* and *P. vivax* infections and have been extensively used in malarial drug and vaccine development. Recently, it has been shown that certain species of *Aotus* can be infected with sporozoites, and that the degree of susceptibility varies among species. We demonstrate here that Panamanian *Aotus lemurinus lemurinus* are susceptible to a sporozoite-induced infection, opening the possibility that this species of *Aotus* could be used as models for testing the efficacy of pre-erythrocytic *P. falciparum* vaccines and drug candidates directed at the pre-erythrocytic stages of *P. falciparum* and *P. vivax* malaria. In this species, we compared sporozoite infection rates. Two of four animals splenectomized prior to infection with sporozoites developed patent parasitemias. Seven of eight animals splenectomized either 7 or 35 days after infection became parasitemic. Additionally, we used a *P. falciparum*-specific polymerase chain reaction (PCR) method to detect the early appearance of parasitized erythrocytes in the blood prior to detection by conventional microscopy, and found that the parasitemia was detected first in five animals by the PCR method, first in three animals by blood film, with one parasitemia detected simultaneously. We also demonstrated the feasibility of infecting monkeys located in Panama with sporozoites isolated at an insectary in Atlanta, thus documenting the feasibility of similar studies where the insectary and monkey colony are not in the same location. A subsequent attempt to infect these monkeys using sporozoites was not successful, suggesting that this model of human malaria is not yet ready for routine use in vaccine or drug efficacy screening. This model merits further study because of the importance of testing pre-erythrocytic *P. falciparum* malaria vaccines and drugs in animals.

Aotus species have long been used as an animal model for infection with erythrocytic stage *Plasmodium falciparum*.¹ Studies in *Aotus* have significantly contributed to the development of anti-malarial drugs suitable for human use, and, relatively recently, served as a model for the development and non-human testing of *P. falciparum* anti-malarial vaccines.²⁻¹³ Although *Aotus* can easily be infected with a variety of adapted erythrocytic strains of *P. falciparum*,¹⁴ only the Santa Lucia strain of *P. falciparum* has consistently induced a sporozoite stage infection in a limited number of species of *Aotus*.¹⁵ Unfortunately the species of *Aotus* (*A. lemurinus griseimembra*) that has been reported to be highly susceptible to a Santa Lucia sporozoite infection is native to northern Colombia and the export of this species has been banned since 1973. Therefore, although an animal model exists for testing the efficacy of anti-malarial vaccines and drugs targeted at the sporozoite stage of *P. falciparum*, the paucity of a highly susceptible species of *Aotus* to a *P. falciparum* sporozoite infection outside of Colombia limits the usefulness of this model. The *Aotus* species indigenous to Panama are *A. lemurinus lemurinus*, and appear closely related to their Colombian counterparts both in phenotype (gray neck species) and in their susceptibility to malaria. Additionally, malaria research using Panamanian *Aotus* as a human malaria model has been conducted for at least 30 years, and historically this species has been available in sufficient numbers for vaccine and drug testing at Gorgas Memorial Laboratory. We report here the susceptibility of Panamanian *Aotus l. lemurinus* to Santa Lucia sporozoite infection.

MATERIALS AND METHODS

Monkeys. Panamanian adult (male and female) *A. l. lemurinus* (karyotype VIII or IX) monkeys¹⁶ were maintained

in the animal facility of the Gorgas Memorial Laboratory 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 pneumoniae*.¹⁷ The animals were housed and cared for as previously described.¹⁷ About one month after arrival, each monkey was tattooed with its identification number and a thick blood film was 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 800 g. All monkeys used in these experiments were wild caught adult monkeys and had no history of experimental infection with *Plasmodium*. Each group of animals within this study had an equal number of males and females.

The experiments reported here 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).

Surgery. Twelve monkeys were used in this study, four were splenectomized before *Plasmodium* infection, four 7 days after infection and four 35 days after infection. Splenectomies were performed as previously described,¹ except that a paramedian rather than a midline approach was used.

Parasites. *Plasmodium falciparum* (Santa Lucia)^{18,19} was

TABLE 1
Infection data for *Plasmodium falciparum* (Santa Lucia) erythrocytic and sporozoite infection in *Aotus lemurinus lemurinus*

Monkey number	Infective dose	Route*	Day of splenectomy	Post-infection period	Days to peak	Peak (parasites/ μ l)	Day of self cure	Reinoculation
12732†	1×10^6	IP	-30	1	26	197,120	47	-
12744†	1×10^6	IP	Not done	1	47	940	54	-
12737	2×10^5	IV	-124	-	-	-	-	-
12736	2×10^5	IV	-124	-	-	-	-	-
12733	2×10^5	IV	-84	14	43	357,090	62	-
12734	2×10^5	IV	-84	14	46	455,820	117	-
12735	2×10^5	IV	-84	14	46	285,660	117	-
12715	2×10^5	IV	-84	14	46	114,120	117	-
12716	2×10^5	IV	-7	21	38	454,040	30	-§
12751	2×10^5	IV	+35	39	72	611,090	-	-#
12750	2×10^5	IV	+35	25	25	<10	26	-
12746	2×10^5	IV	+35	-	-	-	-	-
12747	2×10^5	IV	+35	23	40	154,000	105	+

* IP = intraperitoneally; IV = intravenously.

† Infection induced by transfer of infected blood.

‡ Death on day 91 of non-malaria disorder.

§ Death on day 114 of non-malaria disorder.

¶ Death on day 80 of malaria.

Death on day 76 of malaria.

obtained from the Centers for Disease Control and Prevention (CDC) (Chamblee, GA). Infections were induced by either the intraperitoneal injection of heparinized parasitized erythrocytes (previously frozen) or by intravenous injection of sporozoites dissected from the salivary glands of infected *Anopheles gambiae* mosquitoes. Two monkeys, one splenectomized and one intact, were infected by intraperitoneal injection with 10^6 Santa Lucia strain trophozoites. Sporozoite infection was performed by intravenous injection of 2×10^5 sporozoites dissected from the salivary glands of infected *An. gambiae* mosquitoes as previously described.²⁰ Sporozoite isolation was performed at the CDC Chamblee facility (Chamblee, GA). Once isolated, the sporozoites were transported at room temperature in M199 media containing 5% normal human serum (Gibco Laboratories, Grand Island, NY) directly to the Gorgas Memorial Laboratory in (Panama City, Republic of Panama). The time between the first mosquito dissection and the last monkey injected with sporozoites was 10.5 hr.

Blood films. Daily blood films were prepared for up to 524 days after infection by removing the monkey from its cage and immobilizing it while the marginal ear vein was thoroughly cleaned and pricked with a lancet. From a drop of expressed blood, blood films were made onto pre-cleaned, pre-labeled microscope slides. The slides were stained with Giemsa and parasites/microliter were enumerated by both standard thick film and by assessing Earle-Perez films.²¹ Criteria for enumeration were as follows: a negative parasitemia is reported if no parasites were seen after examining a conventional thick blood film for at least 10 minutes (1,000 fields; 0.67 μ l). If only a single parasite was noted in 160 600 \times fields, an estimated parasitemia of 5 parasites/ μ l was recorded. Parasite enumeration of >10 parasites/ μ l was determined and reported by the Earle-Perez method with 0.1 μ l of blood examined in 160 fields (magnification was 600 \times with oil immersion).

Polymerase chain reaction (PCR) detection of parasites. The PCR was used as previously described to detect *P. falciparum* in these animals.²² One hundred-microliter

blood samples were drawn every other day from day 7 to day 21 and every third day after day 21 for approximately 6 weeks; length of follow-up varied by animal. Extraction of DNA, PCR, hybridization, and an ELISA of the blood samples were performed as previously described²² with the following two exceptions: 1) the blood was collected from the monkeys following marginal ear vein lancet venipuncture in heparinized microcapillary tubes, and 2) extraction of DNA from the blood was performed with the QIA Blood Prep kit (Qiagen, Hilden, Germany).

RESULTS

Trophozoite infection. To demonstrate that *A. l. lemurinus* is a compatible host for the Santa Lucia strain of *P. falciparum*, two monkeys, one splenectomized (12732) and one intact (12744), both without any prior history of *Plasmodium* infection, were inoculated intraperitoneally with 10^6 previously frozen parasitized *P. falciparum* (Santa Lucia) erythrocytes (Table 1). After inoculation, the splenectomized monkey (12732) had a detectable parasitemia (< 10 parasites/ μ l) the day following infection and again on days 10, 11 and 12. On day 17, the parasitemia began to increase and peaked on day 28 with 197,120 parasites/ μ l (Figure 1). On day 48 the monkey self-cured the infection. The non-splenectomized monkey (12744) had only three brief periods of transient parasitemia on day 1, days 5-20 and 40-54 (Figure 2). This monkey's parasitemia was never > 940 parasites/ μ l (day 47) and self-cure of the infection occurred on day 54 (Figure 1).

Sporozoite infection. Having demonstrated that *A. l. lemurinus* is susceptible to infection by blood-stage *P. falciparum* (Santa Lucia) (Figure 1), and that a splenectomized monkey has a higher and longer duration of parasitemia than an intact animal, an experiment was performed to determine if *A. l. lemurinus* can be infected with *P. falciparum* (Santa Lucia) sporozoites. Twelve monkeys were inoculated with *P. falciparum* sporozoites; of these, four were splenectomized prior to inoculation (Figure 2), four monkeys (Figure

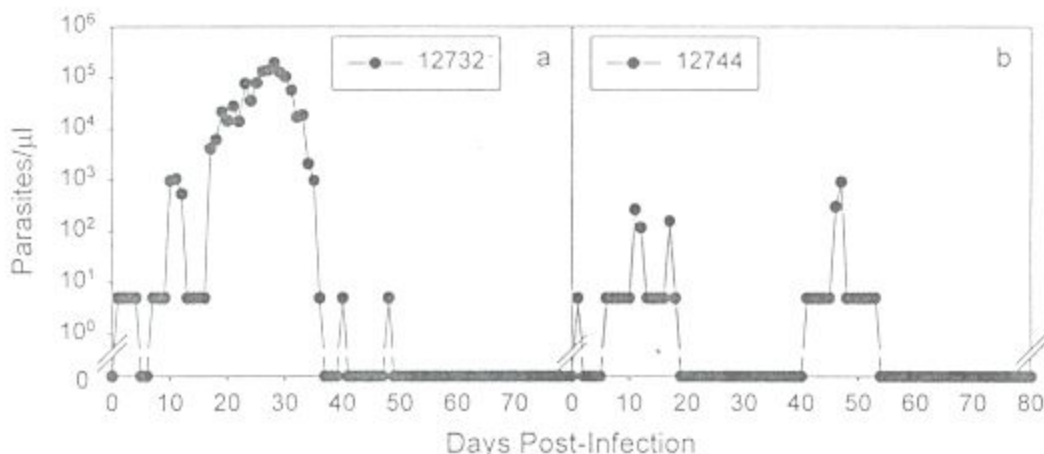


FIGURE 1. Course of infection in two monkeys challenged with 1×10^6 *Plasmodium falciparum* (Santa Lucia) parasitized erythrocytes injected intraperitoneally. Monkey 12732 (a) was splenectomized on day -30 before challenge and monkey 12744 (b) was not splenectomized.

3) were splenectomized 7 days after inoculation, and four were splenectomized 35 days after inoculation (Figure 4).

Of the four monkeys that were splenectomized prior to inoculation, two monkeys (12736 and 12737) were splenectomized 124 days prior to inoculation and two monkeys (12733 and 12734) were splenectomized 84 days prior to inoculation. The monkeys splenectomized on day -124 (12736 and 12737) never developed parasitemias detectable by blood film. The two monkeys splenectomized on day -84 (12733 and 12734) both developed similar parasitemias (Figure 2), and both animals self-cured on days 62 and 182 re-

spectively (Table 1). Monkey 12734 experienced three recrudescences (Table 2) before self-cure.

All four monkeys splenectomized 7 days after infection (12716, 12741, 12743, and 12753) developed parasitemias detectable by blood film (Figure 3). Patency occurred between day 21 and day 29, and 3 of the 4 animals developed dense parasitemias. Monkey 12716 died on day 80 of malaria-related complications. The other three animals self-cured (Table 1). One of these monkeys (12753) had four cycles of recrudescence; the other three did not recrudescence (Table 2).

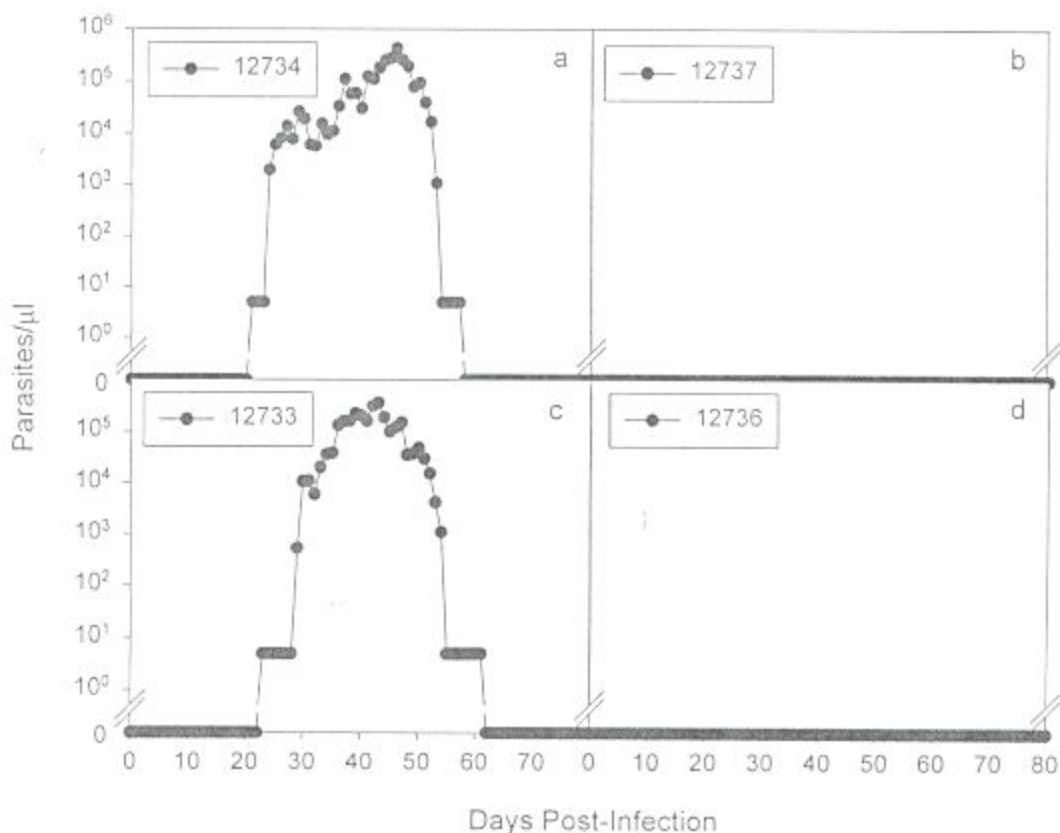


FIGURE 2. Course of infection in four monkeys splenectomized on either day -84 (monkey 12733 and 12734, a and c) or day -124 (monkey 12737 and 12736, b and d) before challenge with 2×10^5 *Plasmodium falciparum* (Santa Lucia) sporozoites injected intravenously.

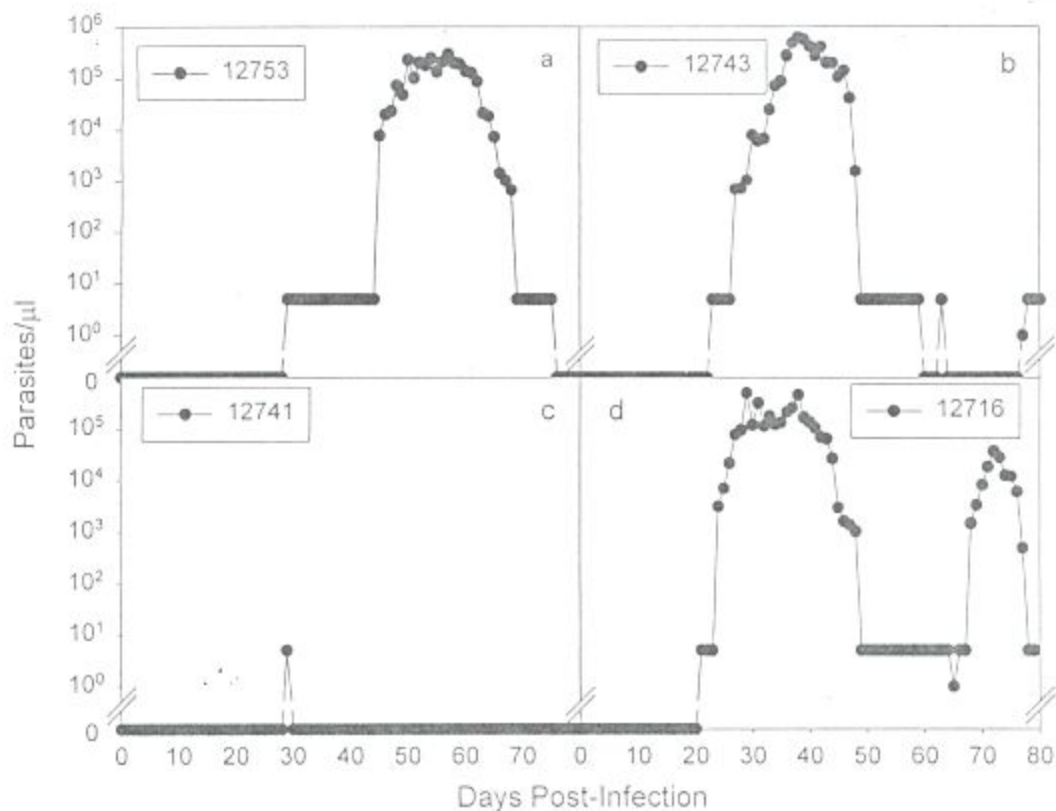


FIGURE 3. Course of infection in four monkeys splenectomized on day 7 after challenge with 2×10^5 *Plasmodium falciparum* (Santa Lucia) sporozoites injected intravenously.

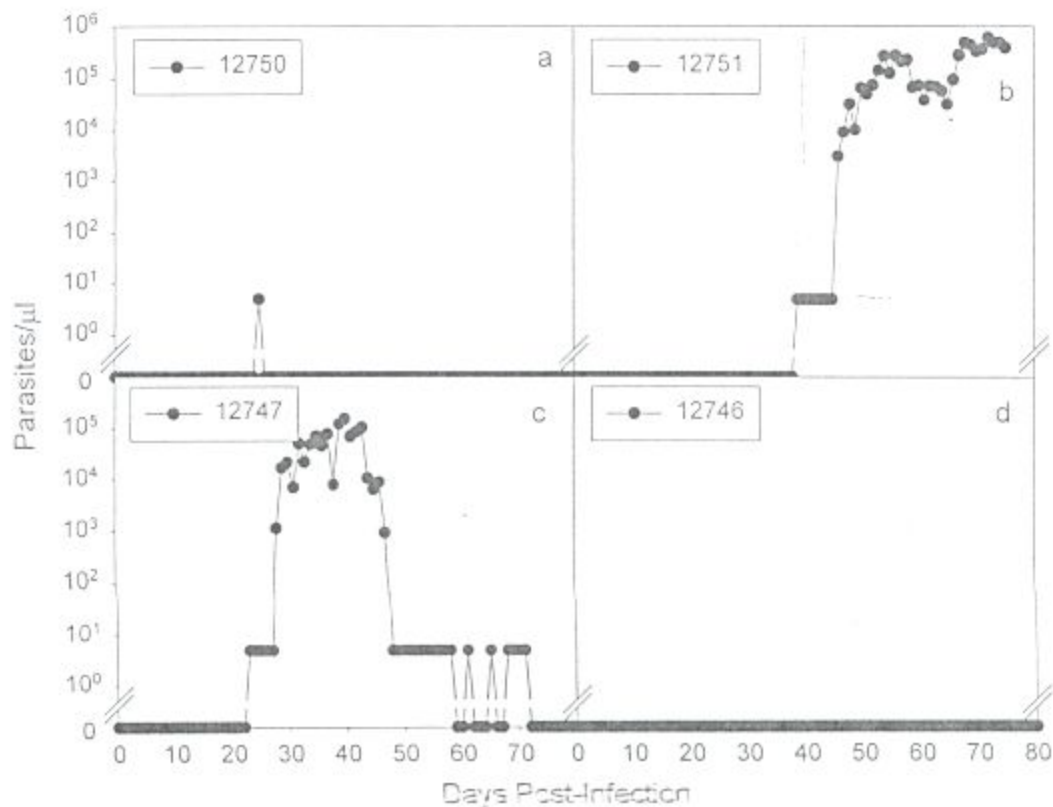


FIGURE 4. Course of infection in four monkeys splenectomized on day 35 after challenge with 2×10^5 *Plasmodium falciparum* (Santa Lucia) sporozoites injected intravenously.

TABLE 2

Summary of recrudescence of *Plasmodium falciparum* (Santa Lucia) sporozoite infection in *Aotus lemurinus lemurinus*

Monkey number	Day of splenectomy	First recrudescence			Second recrudescence			Third recrudescence			Fourth recrudescence		
		Days (# days)	Peak (day)	Peak (per μ l)	Days (# days)	Peak (day)	Peak (per μ l)	Days (# days)	Peak (day)	Peak (per μ l)	Days (# days)	Peak (day)	Peak (per μ l)
12734	-84	84-96 (12)	91	870	127-133 (6)	-	<10	173-183 (9)	-	<10			None
12753	+7	84-107 (23)	92	390	114-135 (21)	-	<10	158-181 (23)	-	<10	221-236 (15)	229	73,140
12747	+35	85-105 (20)	94	418,510		None			None				None

Three of the four monkeys splenectomized 35 days after challenge developed parasitemias detectable by blood film (Table 1). Two of these developed dense parasitemias while one developed a low-density parasitemia detected by blood film only once. Patency occurred between day 23 and day 39. Two of the three parasitemic animals (12750 and 12747) self-cured. Monkey 12747 recrudescence once (day 85) prior to self-cure (Table 2).

Polymerase chain reaction detection of parasites. Concurrent with screening by blood film, we used the PCR to detect parasites in monkey blood. Of the 12 sporozoite-infected monkeys, all were positive at least once by PCR. Nine of 12 were positive by blood film. Although the mean number of days to first positive was 26 days for blood film and 29.5 days for the PCR, in those monkeys positive by both methods, the PCR detected the parasitemia first in five cases (mean = 5.4 days earlier), the blood film detected the parasitemia first in three cases (mean = 12.3 days earlier), and parasitemia was detected simultaneously by both methods in one case. In two of three of the instances where the blood film out-performed the PCR, the parasitemias remained low, never reaching significant densities (monkeys 12741 and 12750).

DISCUSSION

In this study, we have shown that *A. l. lemurinus* can be successfully infected with sporozoites of the Santa Lucia strain of *P. falciparum*. Three different groups of animals were evaluated based on the timing of splenectomy. Two of four animals splenectomized before infection with sporozoites (84 or 124 days prior) became parasitemic. All four animals splenectomized on day 7 after infection became parasitemic, and 3 of 4 animals splenectomized on day 35 after infection also developed parasitemias. All animals developing patent blood-stage infections self-cured. None required drug treatment. The average prepatent periods for the three groups were 22, 25, and 29 days, respectively. The mean peak parasitemias were 395,000/ μ l (range = 357,280-433,250/ μ l) for those splenectomized before infection, 341,000/ μ l (range = 5-616,010/ μ l) for those splenectomized 7 days after infection, and 255,000/ μ l (range = 5-611,090/ μ l) for those splenectomized 35 days after infection. Mean day of peak parasitemia after challenge was 44.5, 40.5, and 45.6 for the groups splenectomized before infection, and 7 and 35 days after infection respectively (Table 1). Two of the monkeys splenectomized on day 35 had already developed patent parasitemias before splenectomy (monkeys

12750 and 12747). One of these animals had a parasite count of 71,000/ μ l on the day of splenectomy. This suggests that although splenectomy may favor the development of parasitemias in animals infected by sporozoite injection, it is not essential in all animals.

Blood films were taken on the animals every day, and blood for the PCR was collected every other day from day 7 to 21, and every third day from day 21 to a subsequent date that varied by animal. Parasitemia was first detected by the PCR eight times compared with 4 times by blood film. When the PCR identified a parasitemia prior to the blood film, it generally did so 2-5 days earlier. Unfortunately, blood samples for the PCR were collected only once every three days to minimize daily stress and handling of the monkeys. The usefulness of the PCR for early detection of parasites in this model may lie in the ability to measure slight differences in the timing of parasitemia onset. If, for example, a pre-erythrocytic vaccine did not provide sterile immunity but instead caused a delay in the onset of parasitemia in immunized monkeys, then this PCR method might have greater power to detect slight differences in the onset of parasitemia between immunized and control monkeys, compared with Giemsa slide blood film. For these reasons, if the PCR is to be used to detect precisely the onset of parasitemia, future studies should include a daily blood sample collection for the PCR until the parasitemia is detectable by a blood film stained with Giemsa.

The prepatency period in those nine animals successfully infected via sporozoite challenge averaged 25 days (range = 21-39 days). The prepatency period of sporozoite-induced *P. falciparum* parasitemia in humans was 11.5 days,²³ although different strains of *P. falciparum* were used (NF54, the 3D7 and CVD1 clones of NF54, and 7G8). The reason for this longer prepatency period in the monkeys is unknown. It is possible that the hepatic stage of parasite development is lengthened, or that development within the hepatocyte is sub-optimal and few merozoites are released upon hepatocyte rupture. It is also possible that the erythrocytic stage grows less efficiently in monkey red blood cells, and more cycles are required to reach a detectable parasitemia.

We have also demonstrated that Santa Lucia sporozoites remain viable and retain their infectivity *in vitro* for at least 10 hr after isolation from the mosquito. Sporozoites used in these studies were produced in *An. gambiae* mosquitoes at the CDC insectary in Chamblee, Georgia, dissected from the mosquitoes, and transported in medium M199 with 5% normal human serum at room temperature to the primate facility

at Gorgas Memorial Laboratory in Panama. The total time between first sporozoite dissection and last monkey injected was 10.5 hr. We have previously reported that *P. yoelii* sporozoites dissected from *An. stephensi* mosquitoes will remain viable and retain infectivity at room temperature at least 36 hr after dissection if kept in M199 media with 5% normal mouse serum.²⁰ We used this method to transport *P. falciparum*, and although not specifically studied, it appeared that the parasites remained viable and did not lose significant infectivity during transport.

In an effort to further validate this procedure as a useful model for malaria vaccine testing, we attempted to repeat this experiment. The 32 animals in this second experiment were vaccinated with either a DNA vaccine encoding three *P. falciparum* pre-erythrocytic stage proteins or the control DNA vaccine containing no *P. falciparum* sequences. None of these animals became parasitemic. This second experiment varied from the first in that the animals were vaccinated and were splenectomized on day 14. These results indicate that while one can successfully infect *Aotus* monkeys with sporozoites of the Santa Lucia strain of *P. falciparum*, this malaria model is not yet reliable enough for routine use in vaccine screening. In spite of this model's present lack of complete reproducibility, it remains worth perfecting because it offers the only current method of testing pre-erythrocytic *P. falciparum* malaria vaccines in non-human primates other than chimpanzees. Other models do exist but only for testing *P. falciparum* blood stage vaccines, or other malaria species (*P. knowlesi* and *P. cynomolgi* for example) in primates.

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