Am. J. Trop. Med. Hyg., 67(5), 2002, pp. 454–458

POTENTIAL OF THE PANAMA STRAIN OF *PLASMODIUM VIVAX* FOR THE TESTING OF MALARIAL VACCINES IN *AOTUS NANCYMAI* MONKEYS

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Abstract. *Aotus* monkeys were infected with a strain of *Plasmodium vivax* from Panama to determine its potential for the testing of malarial vaccines. After sporozoite inoculation, 3 splenectomized *Aotus nancymai* that had been infected previously with *Plasmodium falciparum* and *P. vivax* had prepatent periods of 13, 15, and 15 days with maximum parasite counts of 12,726/μl, 5,310/μl, and 9,180/μl. Three other *A. nancymai* previously infected with *P. falciparum* only had prepatent periods of 17, 15, and 15 days with maximum parasite counts of 44,460/μl, 31,500/μl, and 42,660/μl. One monkey with no previous history of infection had a prepatent period of 14 days after sporozoite inoculation, and a maximum parasite count of 100,000/μl; detectable parasitemia persisted for almost 500 days with 13 recognizable peaks in the parasite count. *Anopheles dirus*, *Anopheles freeborni*, *Anopheles stephensi*, and *Anopheles quadrimaculatus* mosquitoes were readily infected with the Panama strain.

INTRODUCTION

Aotus and *Saimiri* monkeys can serve as models for various studies with human malaria. A major goal is to select animal and parasite combinations for the testing of candidate malarial vaccines. The selection of a usable model for *Plasmodium vivax* has depended on the availablity of appropriate strain and monkey combinations. *Aotus lemurinus grisiemembra* (originating in Colombia) and *Aotus azarae boliviensis* were developed as suitable hosts using different strains of the parasite. These animals are no longer available in the United States. The nonhuman primate *Saimiri boliviensis* was developed as a suitable host for the testing of malarial vaccines directed against the different stages of *P. vivax*.[1–9] Infections with the Salvador I strain of the parasite in monkeys and chimpanzees readily infect different species of laboratory-reared anopheline mosquitoes. Challenge studies of immunized *S. boliviensis* monkeys have been made with sporozoites dissected from infected salivary glands or with infected erythrocytes.

Feral *S. boliviensis* from Bolivia have not been exported for many years, and we have had to rely on laboratory-reared animals. The supply of these monkeys always has been inadequate for extensive studies on the characterization of different isolates of *P. vivax, Plasmodium falciparum*, and *Plasmodium malariae*. To increase the number of suitable models for immunologic and chemotherapeutic studies without depleting the available pool of naive animals, initial identification must rely on the reinfection animals that have been infected previously with heterologous strains and species of malaria parasites.

Feral *Aotus nancymai* imported from Peru could serve as alternate hosts if strains of *P. vivax* could be identified that predictably would induce patent parasitemia after the inoculation of sporozoites or infected trophozoites. When it is established that infections can be initiated via sporozoites and that significant parasitemia can occur, combinations can be developed further in naive animals for the testing of sporozoite vaccines or causal prophylactic drugs. Testing of sporozoite vaccines usually is done with animals that are splenectomized either before or after initiation of development of the liver stages of the parasite.[6,7] Passage of the Vietnam Palo Alto and New Guinea Chesson strains of *P. vivax* in intact *A. trivirgatus griseimembra* (*A. lemurinus griseimembra*) and *A trivirgatus trivirgatus* (*A. nancymai*) has resulted in predictable high-density parasitemia for the testing of blood-stage vaccines and drugs.[10] After 10 serial passages, the parasitemia stabilized; however, gametocyte production was lost. Whether such passage of the Panama strain of *P. vivax* would result in a suitable model for testing blood-stage vaccines in intact *A. nancymai* remains to be shown.

In our attempts to select model systems for the testing of candidate malarial vaccines in *A. nancymai* monkeys, we have initiated a reexamination of strains of *P. vivax* that originally were adapted to *A. l. griseimembra*, or *A. azarae boliviensis* monkeys; some of these strains of *P. vivax* have been stored frozen for many years. Reported here are the results of trophozoite and sporozoite transmission attempts to splenectomized *A. nancymai* using the Panama strain of *P. vivax*.[11,12]

MATERIALS AND METHODS

In the initial studies with the Panama strain between 1966 and 1972, all infections were induced in *A. l. griseimembra* from Colombia. *Aotus nancymai* monkeys, previously infected with different species of *Plasmodium*, were now available for initial attempts to adapt this parasite and to characterize the course of parasitemia. All animals had been obtained commercially or excessed to Centers for Disease Control and Prevention from the Walter Reed Army Institute of Research or the New England Regional Primate Research Center. Splenectomy was done under sterile conditions by a qualified veterinarian. Animals were housed in an American Association for the Accreditation of Laboratory Animal Care, International–approved facility under the direction of the resident clinical veterinarian. Animals were fed a diet of animal chow, fruits, and vegetables shown to be adequate for the maintenance of monkeys in malarial studies.

Monkeys were infected by the intravenous inoculation of parasitized erythrocytes or by the intravenous inoculation of sporozoites dissected from the salivary glands of infected mosquitoes. In an early instance, sporozoites were injected intrahepatically. Beginning 1 day after inoculation of parasitized erythrocytes or 12 days after the inoculation of sporozoites, thick and thin blood films were made by the method of Earle and Perez,[13] stained with Giemsa, and examined microscopically. Parasites were recorded per microliter of blood.

Mosquitoes were laboratory-adapted strains of *Anopheles freeborni* (originally from California), *Anopheles maculatus* (from Malaysia), *Anopheles dirus* (from Thailand), *Anopheles stephensi* (from India), *Anopheles gambiae* (from The Gambia), *Anopheles quadrimaculatus* (from southeastern United States), and *Anopheles albimanus* (from El Salvador). Monkeys were sedated, and the mosquitoes were allowed to feed through the mosquito netting of cages on the clipped abdomens of the animals. The procedures used for feeding, handling, and dissection of the mosquitoes have been presented previously.[14] In other instances, blood was diluted 1:8 in heparinized human blood and fed to mosquitoes through a parafilm membrane. After infection, mosquitoes were held in an incubator at 25 ± 1°C and fed 10% sugar solution on a cotton pad.

Oocyst counts were made microscopically from mosquito guts suspended in a 2% aqueous solution of merbromin (Mercurochrome); this allowed for a contrasting vital staining of the parasites. For collection of sporozoites, the salivary glands were dissected into 20% fetal bovine serum in saline. The glands were crushed under a coverslip, and the sporozoites were washed into a vial. Sporozoites were counted in Neubauer Cell Counting chamber. Sporozoites were injected into the femoral vein of the monkey using a 27G needle.

RESULTS

The Panama strain had been passaged through 5 *A. lemurinus griseimembra* and a human volunteer before being committed to long-term storage (Figure 1). Studies in this monkey host (AO-0157 and AO-0220) indicated that mosquitoes could be infected and that high-density parasite counts occurred after splenectomy (Figure 2 and Table 1). The subsequent passage line was established from a high-density parasite count that occurred during a recrudescence in monkey AO-0220 (Figure 2). Infected erythrocytes from monkey AO-0332 in 10% glycerine had been stored frozen over liquid nitrogen for 10,183 days (>27 years). The blood from AO-0332 was thawed and directly injected into *A. nancymai* monkey AI-2091 (prepatent period, 5 days) and from this animal to AI-2554. Both animals had been splenectomized and previously infected with *P. falciparum*. Their initial heterologous infections had been terminated with chloroquine. Maximum parasite counts for AI-2091 and AI-2554 were 10,260/μl and 92,000/μl on days 29 and 12 (Table 2). The results of subsequent trophozoite and sporozoite passages in *A. nancymai*, 1 *A. vociferans*, and 1 *A. l. griseimembra* are summarized in Table 1. It was apparent that even in splenectomized animals previously infected with heterologous species of *Plasmodium,* relatively high-density asexual parasitemia was produced.

In the early studies, infection of *An. freeborni* and *An. maculatus* was obtained, but only rarely and at low density.

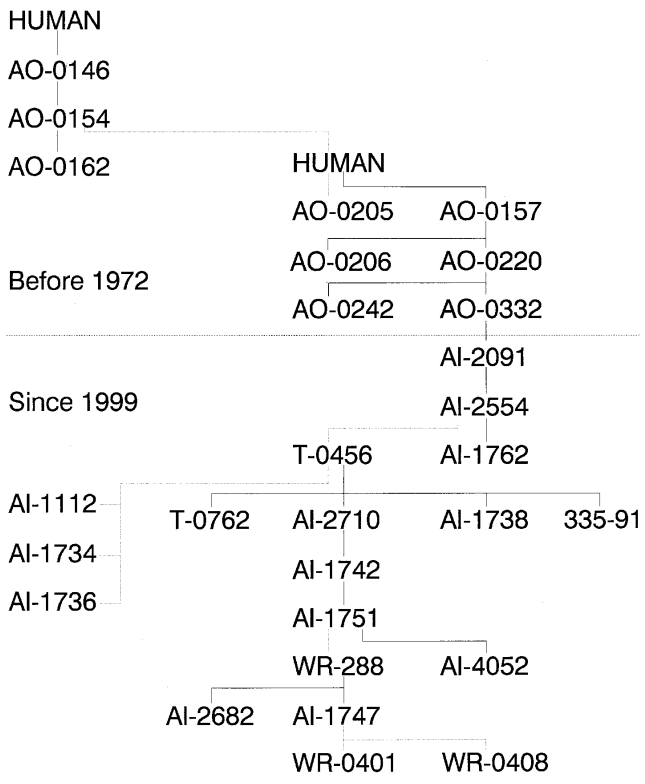

FIGURE 1. Genealogy of the Panama strain of *Plasmodium vivax* since isolation from a patient in 1969. Solid line, trophozoite passage; dotted line, sporozoite passage.

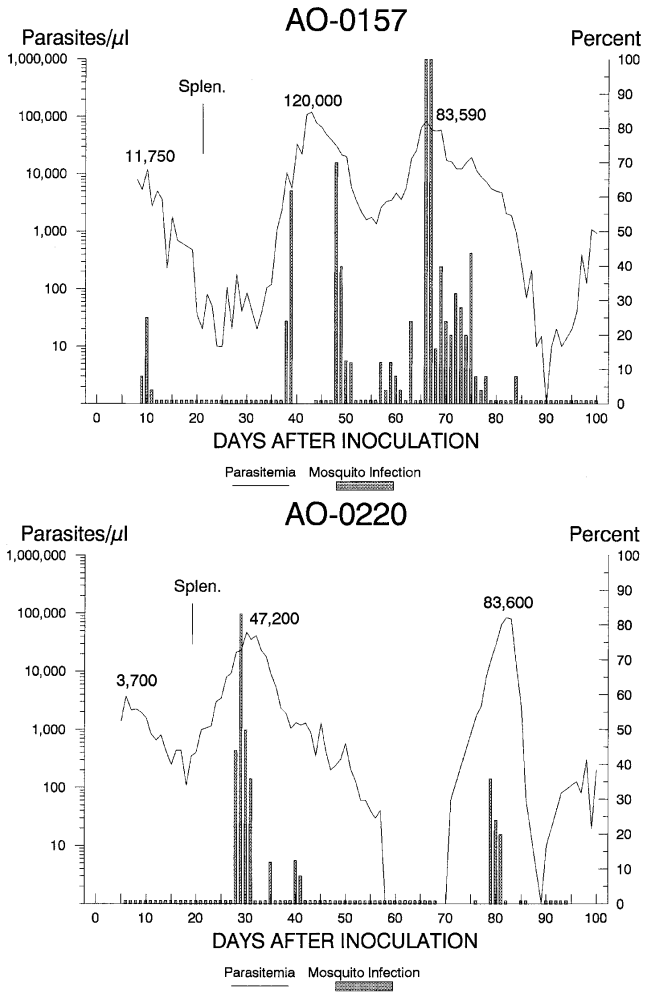

FIGURE 2. Course of asexual parasitemia in 2 *Aotus lemurinus griseimembra*, AO-0157 and AO-0220, infected with trophozoites of the Panama strain of *Plasmodium vivax*. *Anopheles freeborni* mosquitoes were fed to indicate presence of infective gametocytes.

TABLE 1

Previous malaria, inocula, and maximum parasite counts for 28 *Aotus lemurinus griseimembra, Aotus vociferans,* and *Aotus nancymai* monkeys infected with the Panama strain of *Plasmodium vivax*

Passage	Animal	Donor	Monkey species*	Previous infections†	Splenectomy	Inoculum	Maximum parasitemia (day)‡
1	AO-0146	Human	Alg	—	—	?	10,830 (18)
2	AO-154	AO-0146	Alg	—	Day 31	2.1×10^7	46,440 (55)
3	AO-0162	AO-0154	Alg	—	Pre-	$<1.0 \times 10^4$	875 (11)
5	AO-0205	Human	Alg	—	—	81+ glands	2,470 (14)
5	AO-0157	Human	Alg	—	Day 21	?	120,000 (43)
6	AO-0220	AO-0157	Alg	—	Day 27	1.6×10^7	47,200 (30)
							83,600 (51)
6	AO-0206	AO-0157	Alg	—	Day 31	1.0×10^5	10,450 (19)
7	AO-0242	AO-0220	Alg	—	Pre-	4.0×10^8	89,800 (27)
7	AO-0332	AO-0220	Alg	—	Day 30	4.0×10^8	27,990 (14)
8	AI-2091	AO-0332	An	Pv, Pf, Pb	Pre-	8.6×10^6	10,260 (29)
9	AI-2554	AI-2091	Av	Pf	Pre-	2.2×10^6	92,000 (12)
10	AI-1762	AI-2554	An	Pv, Pf	Pre-	3.5×10^6	7,920 (26)
10	T-0456	AI-2554	An	—	Pre-	110,000 Sporo.	100,000 (26)
11	T-0762	T-0456	An	Pf	Pre-	1.9×10^6	64,000 (20)
11	AI-2710	T-0456	An	Pf	Pre-	1.3×10^7	55,800 (8)
11	AI-1738	T-0456	An	Pf	Pre-	1.2×10^7	7,830 (14)
11	335-91	T-0456	An	Pf	Pre-	9.0×10^5	124,000 (19)
11	AI-1112	T-0456	An	Pf, Pv	Pre-	247,000 Sporo.	12,726 (18)
11	AI-1734	T-0456	An	Pf, Pv	Pre-	675,000 Sporo.	5,310 (22)
11	AI-1736	T-0456	An	Pv, Pf	Pre-	1,450,000 Sporo.	9,180 (20)
12	AI-1742	AI-2710	An	Pf	Pre-	1.6×10^6	14,670 (5)
13	AI-1751	AI-1742	An	Pf	Pre-	1.0×10^6	27,990 (13)
14	AI-4052	AI-1751	Alg	Pf	Pre-	6.9×10^5	17,100 (15)
14	WR-0288	AI-1751	An	Pf	Day 6	6,000 Sporo.	44,460 (31)
15	AI-2682	WR-0288	An	Pf	Day 2	1.2×10^7	25,380 (25)
15	AI-1747	WR-0288	An	Pf	Day 2	1.2×10^7	59,580 (13)
16	WR-0401	AI-1747	An	Pf	Day 5	10,000 Sporo.	31,500 (23)
16	WR-0408	AI-1747	An	Pf	Day 5	10,000 Sporo.	42,660 (23)

* Alg, *Aotus lemurinus griseimembra;* An, *A. nancynmai;* Av, *A. vociferans.*
† Pf, *Plasmodium falciparum;* Pv, *P. vivax;* Pb, *P. brasilianum.*
‡ Parasite count/μl of blood; (day) = days after inoculation.

Subsequent studies indicated that early splenectomy greatly increases the production of infective gametocytes.

Mosquitoes were infected by feeding on AI-2554; 110,000 sporozoites were harvested and injected into T-0456, a splenectomized animal not previously infected with *Plasmodium*. The prepatent period was 14 days (Figure 3). The maximum parasite count was 100,000/μl on day 26. The parasitemia in this animal was followed for almost 500 days. During this time, there were 13 recognizable peaks in the asexual parasite count. The intervals between these peaks were 27–56 days. The animal did not receive treatment during this period. Mosquitoes were infected by feeding on T-0456 (Table 2); sporozoites were harvested and injected into *A. nancymai* monkeys AI-1112, AI-1734, and AI-1736. All 3 of these animals had been infected previously with heterologous strains of *P. vivax,* in addition to being infected with *P. falciparum*. An estimated 247,000, 675,000, and 1,450,000 sporozoites were injected (Figure 4A); prepatent periods were 13, 15, and 15 days, and maximum parasite counts of 12,726/μl, 5,310/μl, and 9,180/μl were observed on days 6, 8, and 6 of patent parasitemia. Mosquitoes were infected by feeding on gametocytes from monkey AI-1747 using membrane feeding (Table 2). *Aotus nancymai* monkeys WR-0288, WR-401, and WR-408 were injected with 6,000, 10,000, and 10,000 sporozoites (Figure 4B). These 3 monkeys had been infected previously with *P. falciparum* only. Prepatent periods were 17, 15, and 15 days; maximum parasite counts of 44,460/μl, 31,500/μl, and 42,660/μl occurred on days 15, 9, and 9 of patent parasitemia.

Comparative mosquito feedings were made on monkeys AI-2554, T-0456, and AI-1747 (Table 2). *Anopheles dirus* were the most heavily infected, although all 6 of the currently maintained mosquitoes had oocysts and supported development to the presence of sporozoites in the salivary glands.

DISCUSSION

The Panama strain seems to be well suited to development in *A. nancymai* monkeys. The ready passage via sporozoites to splenectomized monkeys suggests that we may design *P. vivax* vaccine trials in which the challenge can be via trophozoite or sporozoite challenge. The schema for the testing of sporozoite or liver-stage vaccines would be to immunize intact monkeys and splenectomize them when the exoerythrocytic stage development is complete or nearly complete. We believe that the Panama strain and *A. nancymai* monkeys would be suitable for sporozoite challenge of monkeys in the testing of vaccines directed against sporozoites and liver-stage parasites. Whether intact *A. nancymai* monkeys would support high-density parasitemia remains to be determined. In the past, with serial passage, maximum asexual parasite counts increased dramatically, but gametocyte production and mosquito infection were quickly and permanently lost.[11] Two lines would need to be developed: one for sporozoite challenge and one for trophozoite challenge.

The resultant maximum parasite counts in the 3 monkeys previously infected with *P. vivax* and *P. falciparum* were markedly lower than in the 3 monkeys previously infected

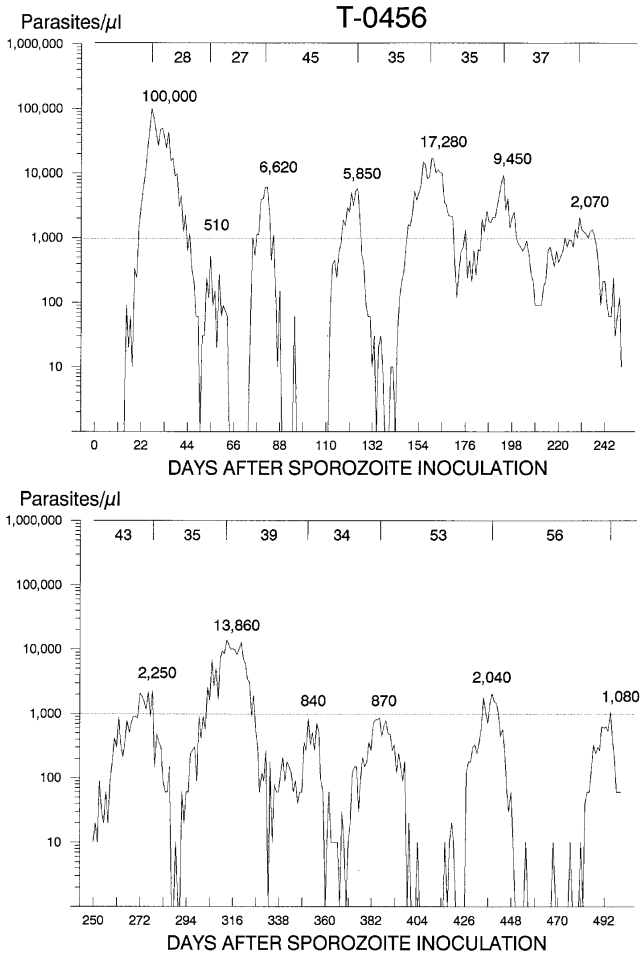

FIGURE 3. Course of asexual parasitemia in *Aotus nancymai* monkey T-0456 infected via sporozoites with the Panama strain of *Plasmodium vivax*. Maximum sequential parasite counts and the number of days between the peak parasite counts are indicated.

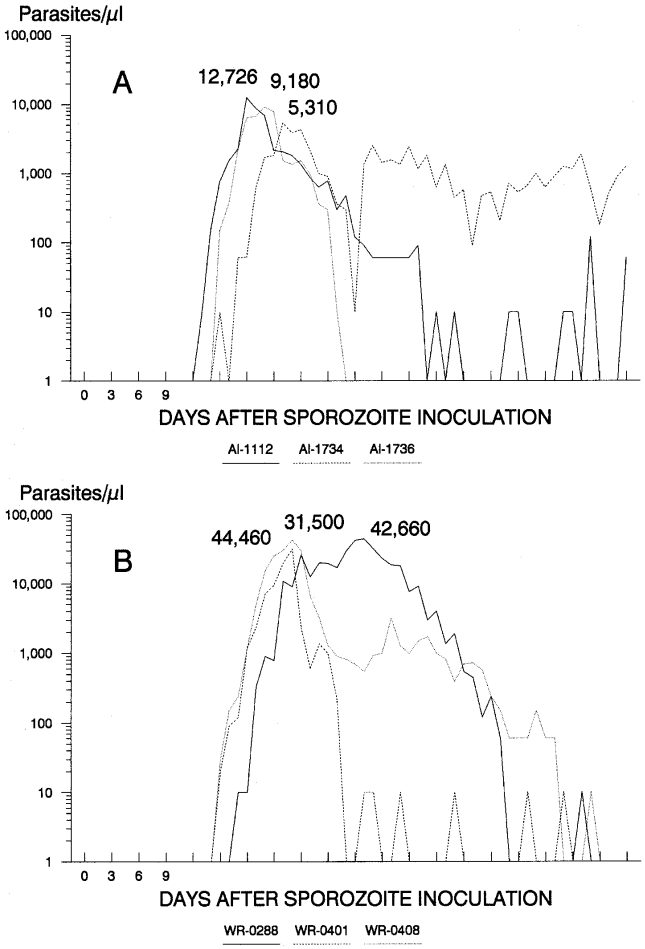

FIGURE 4. **(A)** Course of asexual parasitemia in *Aotus nancymai* AI-1112, AI-1734, and AI-1736 infected with sporozoites of the Panama strain of *Plasmodium vivax*. **(B)** Course of asexual parasitemia in *Aotus nancymai* WR-0288, WR-0401, and WR-0408 infected with sporozoites of the Panama strain of *Plasmodium vivax*.

TABLE 2

Infection of *Anopheles freeborni, Anopheles dirus, Anopheles stephensi, Anopheles gambiae, Anopheles quadrimaculatus,* and *Anopheles albimanus* by feeding on *Aotus nancymai* monkeys infected with the Panama strain of *Plasmodium vivax*

		Species of *Anopheles*											
		freeborni		*dirus*		*stephensi*		*gambiae*		*quadrimaculatus*		*albimanus*	
Monkey	Day*	P/D†	Oo./gut‡	P/D	Oo./gut	P/D	Oo./gut	P/D	Oo./gut	P/D	Oo./gut	P/D	Oo./gut
AI-2554	7	6/10	2.00	11/14	3.79	10/17	2.00	9/24	0.92	9/25	0.64	0/21	0.00
	8	8/13	3.39	11/13	4.62			3/18	0.61				
	9	16/24	9.25	15/22	2.55			2/23	0.26	12/14	10.29		
	10	6/21	0.26										
	11	2/36	0.06										
T-0456	8	13/26	1.46	23/45	1.18	2/23	0.13	4/34	0.12	23/27	8.41		
	9	15/35	1.31	18/18	20.22	17/20	11.20	4/18	0.56	17/28	4.21		
	10			17/21	7.82	27/30	8.20	5/15	2.40	19/24	5.24		
	13	14/14	7.29	18/18	36.83	10/10	19.90			19/28	8.87		
	14	16/16	3.88	13/14	11.57	17/18	7.39			4/25	0.40	10/28	3.14
	15	11/14	6.21	15/15	66.80	21/22	30.14			12/24	3.33		
	16	2/12	0.67	8/24	0.88	16/32	1.19	1/27	0.11				
	23			6/20	0.35			7/33	0.33	2/23	0.09		
	23§							16/45	0.56	18/22	20.82	10/13	11.15
AI-1747	7§	4/35	0.11			0/25	0.00	0/14	0.00	2/25	0.08		
	15§	16/18	2.50			4/30	0.30	0/4	0.00	10/20	1.50	1/32	0.09
	16§	4/14	0.64			1/25	0.04	0/10	0.00	22/26	3.65	5/33	0.50
AI-1747	20§	7/31	0.26			1/31	0.03	1/29	0.03	1/26	0.04	1/16	0.06
	21§	16/24	0.83			1/26	0.04	0/20	0.00	10/17	1.12		
	22§	4/32	0.12			0/33	0.00	2/40	0.05	7/29	0.24		
	23§	10/27	0.89			7/31	0.36			14/23	1.97	0/33	0.00
	56§	3/3	7.00									9/51	0.22

* Day, day of patent parasitemia.
† P/D, mosquitoes with oocysts/number examined.
‡ Oo./gut, oocysts/mosquito guts examined.
§ Blood from monkey diluted 1:8 in heparinized human blood and fed to mosquitoes fed through parafilm membrane.

with *P. falciparum* only. In monkey T-0456, which had not been infected previously, the parasitemia reached high density followed by frequent recrudescences or relapses; it was not possible to determine whether the sequential peaks in the parasite count were the result of the emergence of sequestered parasites from the liver or from the appearance of antigenic variant forms that overcame the host's ability to suppress parasitemia. Nonetheless, the pattern of the parasitemia curve suggests that a splenectomized *A. nancymai* monkey infected with the Panama strain of *P. vivax* may prove useful in studies on the sequential appearance of distinct populations of the parasite whether from relapses or recrudescences.

Of interest was the long-term preservation of this parasite in liquid nitrogen. Additional parasites from monkey AO-0242 (Figure 1) were frozen in 1970 and deposited at the American Type Culture Collection. New stocks of the Panama strain of *P. vivax* are being prepared for deposition.

Financial support: This study was supported in part by Interagency Agreement No. 936-6001 between the U.S. Agency for International Development and the Centers for Disease Conrol and Prevention.

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