Am. J. Trop. Med. Hyg., 40(5), 1989, pp. 465–469 (8-297)

GROWTH AND IMMUNITY CONFERRED BY A *PLASMODIUM FALCIPARUM* TEMPERATURE SENSITIVE MUTANT IN PANAMANIAN OWL MONKEYS

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Abstract. We have compared the growth of the wild type *Plasmodium falciparum* strain Honduras 1 and a previously isolated temperature sensitive mutant of it, AP1-16, in Panamanian owl monkeys. We examined serially infected splenectomized and normal animals that were initially infected with cultured parasites that had been grown in a mixture of owl monkey and human erythrocytes. Initial infections in splenectomized monkeys were marked by multiple recrudescences. The mutant grew less well than the wild type in the splenectomized monkeys, as determined by lower peak and total parasitemias. In the splenectomized monkeys tested by rechallenge with the wild type parasite, the mutant stimulated a comparable degree of protection. That protection was manifested in 2 ways. There was a marked reduction in the level of the primary parasitemia in the rechallenged monkeys and an absence of recrudescent parasitemias after the primary parasitemia. The potential value of generating and studying temperature sensitive *P. falciparum* strains that show attenuated growth is considered.

We previously reported the induction, selection, and isolation from in vitro cultured parasites of temperature sensitive (ts) mutants of *Plasmodium falciparum*.[1] The growth of those mutants was inhibited in the high temperature growth range of the parasite. The goal of that work was the identification of parasites that would, through attenuated growth, be the source of a live vaccine or be used as a tool for probing the relationship between the efficiency of parasite growth and the induction of host immunity. The rationale behind that work was that the growth of a non-reverting ts parasite mutant would be regulated by the febrile response induced by a milder than normal infection. *P. falciparum* infections are characterized by much higher parasitemias than other malaria species[2] and may cause immunosuppression.[3] We reasoned that a temperature regulated infection might provide a low but sufficient level of parasite antigen in an appropriate form to avoid producing an immunosuppressive reaction while inducing a protective immune response. We compared the growth of the wild type *P. falciparum* strain Honduras 1 and a temperature sensitive mutant, AP1-16, that was isolated from it in Panamanian owl monkey.[1] We also compared the effect of parasite growth on the induction of protection to subsequent infection by the same parasite strain.

MATERIALS AND METHODS

Parasites and in vitro parasite cultivation

The *P. falciparum* wild type strain Honduras 1 and 2 ts derivatives were used. The ts mutants were AP1-16, an aphidicolin resistant strain that was also temperature sensitive,[1,4] and QS45,[1] a strain that was directly selected for temperature sensitive growth. The parasites were grown using standard culture methods.[5,6] However, in order to facilitate the growth of parasites in monkey red blood cells (RBC), and therefore in monkeys, the parasites were grown in cultures that contained human A+B+ plasma and both human O+ RBC as well as Panamian owl monkey (POM) RBC.[7] The POM RBC were shipped from Walter Reed Army Institute of Research biweekly and were obtained from POM monkeys of karyotype II and III.[8] We used an ~1:1 mixture of RBC, as we were unable to grow the parasites in cultures for extended periods when they only contained POM RBC.

Infections in Panamanian owl monkey and the monitoring of infections

POM were obtained by and maintained at the Gorgas Memorial Laboratory in Panama.[9] *P. fal-*

ciparum-naive monkeys were used. Splenectomized monkeys were initially infected by iv injection of parasites from cultures. Subsequently, splenectomized or normal monkeys were infected with either fresh, infected monkey RBC or frozen, infected monkey RBC. The transfer of cultured parasites to normal owl monkey can be best achieved by initially passaging the parasites through splenectomized monkeys. Infections were monitored by the daily examination of Giemsa-stained thick and/or Earle-Perez smears. Parasitemias were reported as parasites/mm^3.[9] The parameters used for comparing infections in different animals were: the peak parasitemia (PP) during a period of patency which was determined on Earle-Perez smears; the estimated total parasitemia (ETP) during a period of patent parasitemia, which was calculated as the sum of the daily parasitemias measured in Earle-Perez smears; and the number of recrudescent infections that occurred until there was either a self-cure or drug-induced cure. A self-cure was considered to have occurred after at least 100 days of parasite-negative thick smears following a detectable parasitemia. Infected monkeys that were to be rechallenged after their cure were treated with a single curative dose of mefloquin (30.0 mg base/kg). They were then rechallenged 27 days later with 1.5×10^6 parasites of the wild type Honduras 1 which had been obtained from an infected normal monkey.

RESULTS

Initially the Honduras 1 strain and 2 of its ts mutants, AP1-16 and QS45, were grown in standard in vitro culture conditions for about 1 month with a mixture of human (O+) and POM RBC. Attempts to maintain countable levels of parasites in cultures for several weeks were unsuccessful when the cultures contained only POM RBC. The cultured parasites were then transported to the Gorgas Memorial Laboratory in Panama. Half the parasite cultures were immediately injected iv into splenectomized monkeys. The remainder of each culture was grown for 5 days in vitro at which time each was injected into the appropriate monkey. The sequence of serially infected splenectomized (S) and normal (N) monkeys with the different parasites (Honduras 1 [H], AP1-16 [AP], and QS45 [QS]) is diagrammed in Figure 1 and a comparison of the details of the course and intensity of those infections is provided in Table 1. Parasitized RBC from a successfully infected monkey were either used immediately or frozen and subsequently used to serially infect other monkeys.

We succeeded in infecting splenectomized monkeys 1SH and 1SAP with the Honduras 1 and the AP1-16 strain, respectively, but did not succeed in infecting monkey 1SQS with the QS45 strain. While the course of the successful infections was about the same, an initial parasitemia followed by 3 recrudescent infections over ~300 days, the average PP or ETP was much lower in the AP1-16 infected monkey (see Table 1). The parasites from 1SH and 1SAP were sub-inoculated to normal monkeys (1NH and 2NAP). Since a barely detectable, short duration parasitemia was observed in 2NH and no detectable parasitemia was observed in 2NAP, both monkeys were splenectomized and then successfully infected (2SH and 2SAP, Fig. 1, Table 1). The subsequent parasite growth in 2SAP was, again, much less than in the second Honduras 1 infected monkey. There was also 1 less recrudescent episode in the AP1-16 infected monkey. The less effective growth of AP1-16 in a third set of splenectomized monkeys (3SH and 3SAP, Fig. 1, Table 1) was also observed. The infections were self-cured. We have also tried, without success, to demonstrate that the rates of change of parasitemia caused by the ts mutant AP1-16 were correlated with the monkeys' temperature. Our failure may be because the peak temperatures during infections were either not high enough or not sustained long enough to cause a sharply defined decline in parasitemia rather than a general suppression of parasitemia.

We subsequently serially infected 3 normal monkeys (3NH, 4NH, and 5NH) with the Honduras 1 strain starting with parasites obtained from the second splenectomized monkey (2SH). The initial peak parasitemia in each monkey was 1–2 orders of magnitude lower than that in the splenectomized monkeys. The initial patent periods were followed by a long delay before an even lower peak of recrudescent infection was seen. During the period between the first and second peak of patent parasitemia, long periods (Table 1) of low-level infections (positive only on thick smears) were observed. There was never more than a small second peak of patent parasitemia seen in the Honduras 1 infections of the normal monkeys. The estimated total parasit-

TABLE 1

The course of infection in Aotus *infected with sequentially passaged* P. falciparum

	Parasite Honduras 1				Parasite AP1-16				Parasite QS45		
Monkey	Day*	PP†	ETP‡	Monkey	Day	PP	ETP	Monkey	Day	PP	ETP
1SH	41	400	2,280	1SAP	68	71	701	1SQS	—	0	0
	114	300	1,760		125	37	347				
	167	275	890		179	20	70				
	242	42	220		273	40	57				
2NH	—	0	0	2NAP	—	0	0				
2SH	13	426	4,400	2SAP	8	13	70				
	25	28	58		49	8	30				
	52	30	129		98	26	97				
	98	58	280		211	0	0				
3SH	8	653	4,460	3SAP	10	568	2,849				
	62	50	258		33	39	197				
	107	80	350		247	0	0				
3NH	11	34	105	4NAP	12	57	185				
	89	0.3	1		51	0.6	8				
		+TS§	0		86	1.2	4				
					154	0.9	7				
					230	1.3	4				
4NH	14	1.5	4								
		+TS	0								
	122	0.5	2								
		+TS	0								
5NH	12	9	26								
		+TS	0								
	140	0.5	2								

* Days after the initial inoculation when a PP was observed.
† PP is the peak parasitemia (parasites × 10^3/mm^3).
‡ ETP is the estimated total parasitemia (sum of parasites determined from Earle-Perez smears × 10^3/mm^3 associated with a particular PP).
§ +TS is positive thick smear but not countable on an Earle-Perez smear.

emias was lower than in the splenectomized monkeys (Table 1).

The infection in the single normal monkey (4NAP) with the AP1-16 strain obtained from 3SAP produced an initial PP that was comparable to that in the normal monkey with the Honduras 1 strain (3NH). The initial PP in 4NAP was also comparable to that in the splenectomized AP1-16 infected monkeys 1SAP and 2SAP. Several very low additional recrudescent peaks were seen in 4NAP that were not seen in the infections of monkeys 3NH or 1, 2, or 3SAP (Table 1).

The protection afforded by previous infection in monkeys 1SH, 2SH, 1SAP, and 2SAP was examined by challenging them with 1.5×10^6 Honduras 1 parasites obtained from 5NH (Fig. 1, Table 2). While AP1-16 infections of the splenectomized monkeys produced ~$\frac{1}{5}$–$\frac{1}{25}$ the total number of parasites compared to Honduras 1 during the course of infections in the first 2 infected monkeys (Table 1), all rechallenged monkeys showed only a single peak parasitemia at about the same time and without recrudescent infections (Table 2). The monkeys protected by the less intense AP1-16 infections did, however, show a significantly higher total parasitemia during the parasite rechallenge, suggesting the conferred protection may be related to the intensity of the original infection. In 3 of 4 rechallenged monkeys, the peak parasitemia was lower than that during the original infections. In 1 rechallenged monkey, 2SAP, the peak parasitemia was >10 times higher than that during the initial infection of 2SAP. Monkey 1SQS, which was unsuccessfully infected with QS45, was used as a control. We rechallenged 1SQS with the same dose of 5NH derived parasites. The course of infection in 1SQS was comparable to the original infections caused by Honduras 1 in the parasite-naive monkeys in the high PP and ETP were also seen in the initial and in multiple recrudescent infections (Tables 1, 2). These results suggest that the protection against subsequent infection was a consequence of the initial patent infections of Honduras 1 and AP1-16 and that such protection

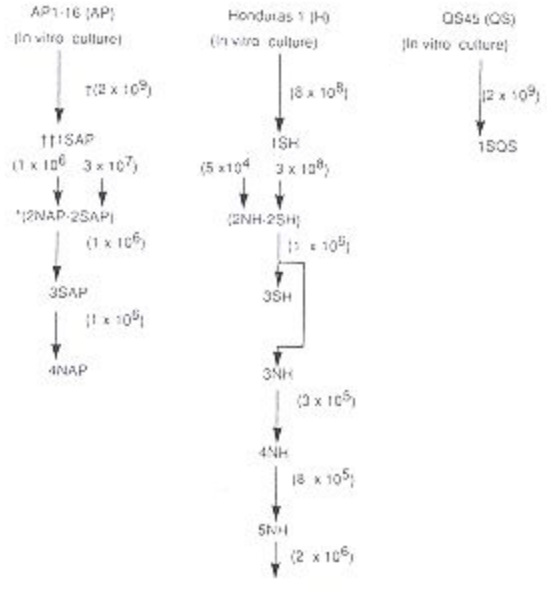

FIGURE 1. A diagrammatic representation of the serial inoculations of Panamanian owl monkey by wild type Honduras 1 and 2 Honduras 1 temperature sensitive mutants AP1-16 and QS45.

* The letters S and N indicate whether the monkey was splenectomized or normal.

† The approximate number of parasites injected.

‡ The number of the monkey passage (inclusive of the monkey) during the serial transfer of the parasite line. The first monkey in each series was inoculated with an in vitro parasite culture that contained both human and monkey RBC.

** Previously infected and cured monkeys rechallenged with parasites obtained from monkey 5NH.

could be achieved by the markedly reduced level of AP1-16 infection.

DISCUSSION

The goals of these studies were to establish the growth of *P. falciparum* Honduras 1, AP1-16, and QS45 in splenectomized and, subsequently, non-splenectomized Panamanian owl monkeys; to determine if a ts mutant causes an attenuated infection; and to determine if a significantly reduced growth of a ts mutant in monkeys would provide protection, comparable to the wild type, against a subsequent infection with the wild type.

We observed that the *P. falciparum* mutant AP1-16, which is both resistant to aphidicolin and exhibits temperature-sensitive growth, produced lower infections (1–2 orders of magnitude) in the splenectomized Panamanian owl monkey than did the wild type strain, Honduras 1, from which it was isolated. As the AP1-16 and Honduras 1 strains grew equally well in in vitro culture, we presume the poorer growth of AP1-16 in monkeys is associated with the temperature sensitive growth of the mutant. This mutant, in spite of its much poorer growth, conferred on its hosts a degree of protection against subsequent Honduras 1 infection, as measured by inhibition of recrudescent infections, that was comparable to that conferred by the wild type. The results did suggest the possibility of parasite mutant reversion. We do not know if the somewhat increased growth of AP1-16 in the monkey 3SAP and the failure to see a lower level of AP1-16 growth in 4NAP than was produced by Honduras-1 in 3NH represented a reversion of AP1-16 towards wild type growth. In general, however, we consider these preliminary results to be supportive of the idea that it is worthwhile to develop ts mutants as tools for combating and manipulating malaria parasites. This limited success prompts the question of whether the further isolation of ts mutants should continue using the tedious and inefficient parasite cloning system.[1-4] It may prove more efficient to induce and select non-reverting ts malaria mutants by constructing them from cloned and sequenced genes

TABLE 2

The course of infection in cured Aotus *challenged with the Honduras 1 strain*

Monkey	Day	PP	ETP	Monkey	Day	PP	ETP	Monkey	Day	PP	ETP
1SH	13	13	65	1SAP	12	40	207	1SQS	8	35	125
2SH	9	43	166	2SAP	13	166	756		30	310	1,796
									50	94	29
									60	26	141
									73	50	266
									150	0.1	0.3
									226	1.1	3.5

All monkeys were inoculated with 1.5×10^6 parasites obtained from monkey 5NH noted in Fig. 1 and Table 1.

that encode known essential parasite proteins. This might be facilitated by the application of in vitro mutagenesis techniques to such genes and by the use of various gene transformation systems including the development of a parasite gene transformation system.

Acknowledgments: We thank Robert Beattie of WRAIR for providing POM RBC and the staff of the Gorgas Memorial Laboratory for their generous assistance.

Financial support: National Institutes of Health, grant AI 020437.

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