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Plasmodium knowlesi: Persistence of Transmission Blocking Immunity in Monkeys Immunized with Gamete Antigens

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Eight rhesus monkeys immunized with a partially purified preparation of *Plasmodium knowlesi* gametes were monitored for over 6 years to determine the extent of transmission blocking immunity. Monkeys were challenged regularly, and anti-gamete antibodies were assayed by in vivo and in vitro mosquito feedings. Transmission blocking immunity persisted at high levels in most of the monkeys. In those animals in which protection waned between challenges, a challenge infection provided a sufficient booster effect to prevent infection of mosquitoes. Immunity to other stages of malaria (i.e., sporozoites and asexual erythrocyte forms) failed to induce immunity against gametes.

Antigens derived from the sexual stages of malaria parasites can induce transmission blocking immunity in a variety of animal hosts, including chickens (2, 5), monkeys (6, 7), and mice (8, 10). Immunity operates by affecting the development of the malarial parasite within the gut of the mosquito vector. Mosquitoes fail to become infected after ingesting gametocytes from an immunized host, and transmission of the parasite is prevented.

Monoclonal antibodies against the surface antigens of gametes have been produced which suppress the infectivity of *Plasmodium gallinaceum* (7a, 12) and *Plasmodium falciparum* (13) to mosquitoes.

Transmission blocking immunity against the sexual stages of *Plasmodium knowlesi* was induced in rhesus monkeys after inoculation with 10^7 or fewer microgametes (7). Immunity lasting up to 1.7 years has been reported (6), but the persistence of long-term protection has not been explored. In this study, we examined the extent of transmission blocking immunity, various factors influencing its induction, and the boosting effect of infection on immunity against the sexual stages of the malaria parasite.

MATERIALS AND METHODS

Macaca mulatta rhesus monkeys of both sexes, weighing 3 to 6 kg at the time of immunization, were used in all experiments. Animals were individually caged in rooms with constant temperature (24 ± 2°C) and relative humidity (60 ± 5%) with illumination between 6 a.m. and 6 p.m.

Malaria parasites. Two strains of *P. knowlesi* were used in this study, the H strain from Malaysia (4) and the P strain from the Philippines (9). All immunization protocols used the $SICA^-$ (Schizont infected cell aggluttination) phenotype of these strains (1), although the precise nature of variability of the SICA phenotype was not known when these studies were initiated.

Monkeys were usually challenged with $SICA^-$ parasites, although in one series of challenges, a more virulent $SICA^+$ variant of the H strain was used. In another experiment, trophozoites of the W-1 variant of the H strain (11) were used for immunization; this variant does not produce gametocytes and cannot infect mosquitoes.

Immunization regimens. Most of the monkeys in this study had been immunized as previously described (7). The typical antigen preparation consisted of 10^5 to 10^7 microgametes and lesser numbers of macrogametes and asexual parasites emulsified in Freund complete adjuvant (FCA). This mixture was inoculated intramuscularly, and monkeys received one or two inoculations before being challenged.

A second group of monkeys was immunized with various stages of *P. knowlesi* parasites to test the effects of other stage-specific antigens on transmission blocking immunity. Two monkeys each received 10^9 trophozoites of the W-1, gametocyteless variant of *P. knowlesi* in FCA. Three monkeys were immunized with *P. knowlesi* merozoites in FCA. One animal was immunized by repeated intravenous inoculation with *P. knowlesi* sporozoites without adjuvant.

Challenge infections. After the initial 2-year study, monkeys were challenged annually for up to 6 years. The standard challenge inoculum of 10^5 schizonts of *P. knowlesi* was given intravenously. Parasitemia was monitored by taking daily blood samples that were examined as Giemsa-stained thin smears. Animals in which the infection showed a rapid rise in parasitemia were chloroquine treated when at least 5% of their erythrocytes were infected. Self-limited infections were drug treated 3 to 4 days after the peak of parasitemia. Serum was prepared from blood drawn before and after each challenge infection.

Assays for transmission blocking immunity. Transmission blocking anti-gamete immunity was tested in vivo by feeding *Anopheles dirus* mosquitoes on monkeys for which challenge infections rose above 0.1%. Feeding was accomplished by applying caged mosquitoes to the shaved abdomens of immobilized monkeys; mosquitoes readily fed and became engorged with blood through the screening of the cage. Mosquitoes were dissected 7 days after feeding, and oocysts growing on the gut were counted. Immunity was indicated by a reduction in oocyst number in mosquitoes fed on immunized monkeys as compared with oocysts found in mosquitoes that had fed on control animals with similar parasitemias.

Serum from immunized animals was tested for antigamete transmission blocking immunity as follows. Gametocyte-containing blood drawn from a *P. knowlesi*-infected monkey

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TABLE 1. Effects of various prechallenge treatments on the long-term course of asexual parasitemia and infectivity to mosquitoes of *P. knowlesi* infections in rhesus monkeys

Monkey no.	Treatment	Total infections		Oocysts per gut at last challenge infection		Oocysts per gut (mean of all direct feedings)
		Spleen intact	Postsplenectomy	Direct feeding on monkey	Membrane feeding[a]	
298	Repeatedly infected control	5	4	≥80	≥100	≥100
420	Repeatedly infected FCA + serum pretreatment	1	10	≥100	≥100	≥100
730	Merozoites + FCA[b]	5	0	No feedings	≥70	No feedings
613	Merozoites + FCA[b]	5	0	No feedings	≥100	No feedings
618	Merozoites + FAC[b]	5	0	No feedings	≥150	0
726	Trophozoites + FCA[c]	3	3	≥130	≥100	≥80
725	Trophozoites + FCA[c]	1	1	≥50	≥80	≥71
89	Sporozoites[d]	6	0	≥100	≥100	≥100

[a] Serum was drawn after the last challenge infection, mixed with parasites from donor monkey, and fed to mosquitoes through membrane.
[b] Monkeys were immunized with three intramuscular injections of 10^9 *P. knowlesi* merozoites emulsified in FCA. Challenge of intact monkeys resulted in no detectable parasitemia.
[c] Monkeys were immunized with two intramuscular injections with 10^9 *P. knowlesi* trophozoites emulsified in FCA. Challenge of intact monkeys resulted in maximum parasitemias of less than 0.5%.
[d] Monkey was immunized with 11 intravenous injections of 10^7 *P. knowlesi* sporozoites and was completely resistant to challenge by bite of infected mosquitoes. After challenged by injection of blood-stage parasites, fatal *P. knowlesi* infection developed.

was washed in saline, resuspended in serum, and fed to mosquitoes through a membrane feeding device consisting of a membrane-covered, water-jacketed (37°C) chamber (14). Mosquitoes ingested resuspended blood through the membrane much as they would ingest blood from a monkey. The antigamete activity of the serum was determined by the reduction in the number of oocysts in mosquitoes fed on the suspension in test serum as compared with the numbers of oocysts developing on the guts of mosquitoes fed on a suspension of infected erythrocytes from the same monkey in normal serum.

RESULTS

Monkeys repeatedly infected by blood inoculation. Rhesus monkeys showed a characteristic response to repeated cycles of infection and cure, both in the severity of infection with asexual parasites and the infectivity of gametocytes to mosquitoes. The abbreviated infection histories of two control animals are shown in Table 1. One of these (monkey 420) had been pretreated with FCA to determine whether the adjuvant had any nonspecific effects on the development of immunity. After four or five challenge infections with *P. knowlesi*, spleen-intact monkeys developed sufficient immunity to limit parasite development, and only low-grade or subpatent parasitemias resulted. Few gametocytes were produced by these low-grade parasitemias, and little mosquito infection took place. However, when the spleen was surgically removed, these animals lost their ability to suppress parasite growth. Splenectomized monkeys could be infected repeatedly. Each challenge produced a rapidly developing, potentially lethal infection, and these monkeys were highly infectious to mosquitoes. Only rarely did a splenectomized animal again demonstrate immunity to asexual erythrocytic parasites.

Serum from repeatedly infected control monkeys prepared from blood drawn either before or after an infection failed to show any evidence of transmission blocking antigamete antibodies.

Monkeys immunized with various antigens other than gametes. Table 1 presents infection histories of monkeys immunized with merozoites, trophozoites, and sporozoites of *P. knowlesi*. Stage-specific immunity was produced by all three antigens. Immunization with merozoites or trophozoites produced immunity to the asexual stages of the parasite; upon challenge, little or no parasitemia was demonstrable. However, transmission blocking antibodies could not be shown in either group of monkeys. Serum from these animals had no effect on the capacity of gametocytes to infect mosquitoes.

One monkey developed sterile anti-sporozoite immunity after repeated intravenous inoculation with sporozoites of *P. knowlesi*. This animal was resistant to challenge with sporozoites of *P. knowlesi*. When challenged by intravenous inoculation of asexual parasites, parasitemia developed rapidly and gametocytes were highly infectious to mosquitoes. Transmission blocking immunity did not result from the use of sporozoite antigens.

Monkeys immunized with gamete antigens. Eight monkeys immunized in 1976 and 1977 were monitored for over 6 years until the final challenge infection in 1983 (Table 2). The first three monkeys were splenectomized soon after immunization and repeatedly challenged thereafter. Of these animals, two (monkeys 442 and 462) remained susceptible to challenge throughout the study; each postsplenectomy infection developed rapidly and required drug treatment. One of this group, monkey 533, was able to control its infection on the seventh postsplenectomy challenge and showed no parasites after the subsequent three challenges.

In these three monkeys, transmission blocking immunity persisted throughout the 6-year course of the study. Few oocysts developed in mosquitoes fed directly on these infected monkeys in spite of frequently high parasitemias, significant numbers of sexual parasites, and demonstrable exflagellation. Exflagellation of microgametocytes in a nonimmunized monkey is a good indicator of infectivity of those gametes to mosquitoes.

Five monkeys were challenged over a 5-year period with their spleens intact. Of these, only monkey 147 developed patent infections after the later challenges, and only this monkey continued to infect mosquitoes, albeit at a level far below that of control animals. Four of this group were splenectomized in 1983 and challenged. All four developed patent parasitemias, and three required chloroquine treatment. In monkeys 114, 63, and 147, parasitemia developed rapidly, and male gametocytes were readily exflagellated.

TABLE 2. Effects of various immunization regimens with gamete antigens and repeated challenge on the course of asexual parasitemia and infectivity to mosquitoes of *P. knowlesi* in rhesus monkeys

Monkey no.	Immunization regimen		Total challenge infections		Last challenge infection (February 1982)			Oocysts per gut (mean of all direct feedings)
	Date started	Microgametes + FCA	Spleen intact	Postsplenectomy	Maximum parasitemia %	Oocysts per gut		
						Direct feeding	Membrane feeding[a]	
533	April 1976	10^7	1	9	0	No feeding	0	0.09
442	June 1976	10^7	1	6	2[b]	0	0	0
462	February 1977	10^5 (2×)	2	7	4[b]	5	0	1.2
114	February 1976	10^6 (2×)	8	1	1[b]	0	0	0
434	April 1976	10^7	11	1	0.01	No feeding	0	0
63	March 1977	10^6 (2×)	8	1	11[b]	0	0	0
147	January 1977	10^5 (2×)	9	1	9[b]	2.5	9	4.4
474	February 1977	10^5	6	0	0	No feeding	0	0
298		Control	5	4	7[b]	≥80	≥100	≥100

[a] Serum was drawn after last challenge infection, mixed with parasites from donor monkey, and fed to mosquitoes through a membrane.
[b] Monkey treated with chloroquine at parasitemia to prevent death.

Transmission blocking activity could be demonstrated in the serum of all five monkeys. Four showed complete blockage, whereas serum from monkey 147 was able to reduce oocyst development by more than 80%.

Booster effects of infection on transmission blocking immunity. In some immunized monkeys, transmission blocking immunity appeared to wane with time (Table 3). Serum prepared before challenge from monkeys 147 and 462 often failed to block infection in vitro, whereas serum drawn 1 to 2 weeks after challenge showed high levels of protection in the in vitro membrane feeding test. At the same time, direct feedings on these monkeys during the course of patency indicated that their gametocytes were barely, if at all, infectious to mosquitoes and that transmission blocking antibodies were present at the time of feeding. In two other monkeys (63 and 533), high antibody levels persisted between challenges.

The challenge infection itself appeared to boost transmission blocking immunity levels sufficiently to prevent mosquito infections by the time infectious gametocytes appear in the circulation. Although antibody levels were low before challenge in some of these monkeys, levels rose during the prepatent period and transmission blocking effects were apparent when parasitemias were high enough to allow for mosquito feedings.

DISCUSSION

In this study, monkeys were initially immunized with one or two injections of an antigen mixture consisting primarily of microgametes, with lesser numbers of macrogametes and asexual trophozoites. The resulting antibodies were able to block development of the malaria parasite within the gut of the mosquito vector and effectively prevent transmission of the parasite from host to host.

Immunity persisted for at least 6 years in most of these

TABLE 3. Booster effect of challenge infections on transmission blocking immunity at various times after immunization

Monkey no.	Immunization regimen (microgametes in FCA)	Challenge infection			Maximum parasitemia %	Oocysts per gut			
		Spleen intact	Postsplenectomy	Yr after immunization		Direct feeding	Membrane feeding[a]		
							Control	Prechallenge	Postchallenge
63	10^6 (2×)	5		2	3	0	≥100	0	0
		6		3	0.7	0	≥100	0	0
		7		4	0	No feeding	≥50	0	0
		8[b]		6	0	No feeding	≥100	0	0
			9	6.5	11[c]	0	≥80	0	0
147	10^5 (2×)	6		2.5	4	0	≥130	≥170	0
		7		3.5	1.7	0	≥100	≥100	0.6
		8[b]		4	0.5	0	≥100	22	3
			10	6	9[c]	2.5	≥80	18	9
533	10^7		7	3	2	0.8	≥100	0	0
			8	4	0	No feeding	≥100	0	0
			9	4	0	No feeding	≥50	0	0
			10[b]	5	0	No feeding	≥100	0	0
462	10^5		6	2.5	8[c]	0	≥100	≥100	0
			7	3.5	17[c]	0	≥100	0.4	0
			8[b]	4	13[c]	6	≥100	≥80	0
			9	6	4[c]	5	≥100	≥50	0

[a] Serum was drawn before and after challenge infection, mixed with parasites from donor monkey, and fed to mosquitoes through a membrane.
[b] Monkey challenged with $SICA^+$ variant of the H strain of *P. knowlesi*.
[c] Monkey treated with chloroquine at parasitemia indicated to prevent death.

animals, although probably not without the help of regular challenge infections which served to boost immunity. In rhesus monkeys, repeated infection with *P. knowlesi* induced immunity against the asexual stages of the parasite. However, repeated infection did not appear to induce the production of antibodies against the sexual stages; there was no evidence of transmission blocking immunity in these monkeys. These findings are in agreement with the results from human subjects in endemic areas chronically infected with *P. falciparum*. These individuals show immunity to asexual parasites, but not to the sexual stages (3).

The role of the infection in boosting gamete-specific immunity is unclear. In spleen-intact, gamete-immunized monkeys, repeated challenges produced little or no parasitemia, yet protection against gametes persists. At the same time, in those animals in which protection waned, a challenge infection was sufficient to boost immunity and prevent the infection of feeding mosquitoes. Gametocytes possess a number of antigens in common with gametes (12, 13), and gametocytes developed during the challenge infection could contribute to boosting of anti-gamete immunity. Less likely, antigens common to both sexual and asexual stages might be involved. The challenge inoculum itself contained gametocytes, but in numbers (usually less than 10^3 per injection) insufficient to provide a significant boost. It must be remembered that repeatedly infected, but nonimmunized, rhesus monkeys suffer high parasitemias with significant numbers of gametocytes in circulation but fail to develop antigamete immunity.

The booster effect of a malarial infection could prove to be particularly important in the maintenance of transmission blocking immunity against *P. falciparum*. Gametocytes begin to appear in the circulation 7 to 10 days after the first asexual parasites, and the peak of sexual parasitemia in *P. falciparum* infections comes well after the peak in asexual parasitemia. Each *P. falciparum* infection could effectively boost immunity against later-forming sexual parasites.

A secondary, but by no means insignificant, feature of the initial immunization scheme was the induction of immunity to asexual parasites. As originally reported by Gwadz and Green (7) and confirmed in this study, monkeys immunized with the partially purified mixture of microgametes, macrogametes, and trophozoites not only produced antigamete antibodies but were able to significantly suppress an otherwise normally fatal infection of *P. knowlesi*. It would appear that this immunity against the asexual infection was induced by trophozoite antigens. Indeed, as previously noted, a preparation of pure trophozoites in FCA was effective in inducing immunity against asexual infection without inducing gamete-specific immunity. The capacity of gamete antigens to induce antibodies against asexual parasites remains to be determined.

The requirement for a potent adjuvant for immunization of monkeys with gamete antigens has been considered a serious drawback. However, Mendis and Targett (10) have been able to immunize mice with 6×10^7 gametes without adjuvant. Given that a rhesus monkey may weigh at least 200 times more than a mouse, it is not inconceivable that a significant increase in the amount of gamete antigen might induce immunity without the need for adjuvants. Antigen synthesis or recombinant DNA techniques should produce antigenic material sufficient to test this hypothesis.

A vaccine designed to induce transmission blocking immunity would be an altruistic vaccine; antigamete antibodies would confer no direct benefits on the immunized individual. However, a trivalent or polyvalent vaccine with antigens of sporozoites, asexual stages, and gametes could produce a level of immunity which could significantly alter morbidity, mortality, and patterns of transmission of malaria in the human population. The feasibility of such a vaccine remains to be demonstrated.

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