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Boosting of Transmission-Blocking Immunity during Natural *Plasmodium vivax* Infections in Humans Depends upon Frequent Reinfection

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The infectivity to mosquitoes of 31 acute *Plasmodium vivax* patients was measured by permitting mosquitoes to feed directly on the patients. The infectivity of these patients correlated closely with titers of antibodies in their serum as measured by indirect immunofluorescence against air-dried female gametes of *P. vivax*. Infectivity by direct feeding was also closely parallel to the transmission-blocking activity of the sera of patients as measured by the suppression of infectivity of parasitized blood by autologous serum relative to normal (nonmalarial) human serum when fed to mosquitoes through a membrane. These results are consistent with serum antibodies in human *P. vivax* infections as major factors determining the infectivity of an infected individual to mosquitoes. It was further noted that individuals having a second attack of *P. vivax* within less than 4 months were considerably less infectious to mosquitoes than first-attack patients were. This "boosting" of transmission-blocking immunity was much less if longer intervals intervened between attacks. We discuss the immunological implications and possible epidemiological significance of this short-term boosting of transmission-blocking immunity by successive *P. vivax* infections.

Following repeated malaria infections over several years, individuals living in malaria-endemic regions develop immunity against the pathogenic asexual blood stages of the parasite; such immunity reduces parasite loads and the severity of the clinical illness (2). Natural malaria infections also induce immunity against sporozoites, the stages which are inoculated through the bite of an infective mosquito (3, 9), which may enhance protection against malaria. The effect of such naturally acquired immunity to malaria on infectivity of patients was thought to be only indirect, by lowering parasitemias and hence gametocyte counts. Recently we demonstrated the occurrence of naturally acquired antimalarial immunity directed specifically against the sexual stages, gametes, which develop in the mosquito vector and effect transmission of the disease (7); antigamete antibodies develop readily in response to natural vivax malaria infections and, when taken up in the blood meal of a mosquito, neutralize gametes and suppress the development of the parasite in the vector, thus reducing transmission of malaria.

Our previous study (7) demonstrating naturally occurring transmission-blocking immunity in humans was done by feeding each patient's blood cells, suspended in his own (autologous) or nonmalarial (control) serum, to mosquitoes through a membrane. In the present study we fed mosquitoes directly on patients in an attempt to measure the true infectivities of the patients to mosquitoes and to correlate this with serological assays of antigamete antibodies and serum-mediated transmission-blocking factors. We have also made further observations on the effects of previous malarial infections on the infectivity of *Plasmodium vivax* patients to mosquitoes.

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MATERIALS AND METHODS

Patients. After voluntary informed consent was obtained, 31 consecutive unselected gametocyte carriers among acute vivax malaria patients presenting to the General Hospital, Colombo, were investigated in this study. A history of the past and present experience with malaria was obtained from each patient; in most cases the history was confirmed by previous hospital records, and in others the histories were validated by the fact that all patients were literate and reliable informants (4).

All patients in this study received a full curative regimen of antimalarial chemotherapy after diagnosis and initial withdrawal of blood for the experiments described here.

Sera. Serum was prepared from each patient and stored at −20°C or used fresh for performing infectivity assays. Normal (nonmalarial) group AB human serum (NHS) was used in controls.

IFT. The indirect immunofluorescence test (IFT) was performed as previously described (7), using air-dried smears of *P. vivax* female gametes as the antigen. Briefly, dilutions of sera in phosphate-buffered saline were incubated for 30 min at room temperature, rinsed in phosphate-buffered saline, and incubated with a 1:20 dilution of fluorescein-conjugated anti-human immunoglobulin (Cappel Laboratories, Cochranville, Pa.) for 30 min. The slides were rinsed again in phosphate-buffered saline, overlaid with a glass cover slip, and examined by UV light in a Leitz Ortholux microscope.

Assay for the infectivity of *P. vivax* parasites to mosquitoes. *Anopheles tessellatus*, a species indigenous to Sri Lanka, which is being maintained as a laboratory colony, was used as the mosquito vector in this study. In direct feeding experiments, approximately 20 3- to 4-day-old female mosquitoes contained in a paper cup were allowed to feed on the patient's forearm for 15 to 20 min. Simultaneously, 4 ml of parasitized blood from the patient was drawn without anti-

coagulant and prepared for membrane feeding to mosquitoes as follows. Each sample (2 ml) of venous blood was diluted immediately in 10 volumes of a suspended activation solution (10 mM Tris, 170 mM NaCl, 10 mM glucose, pH 7.4) in which gametocytes could be maintained for a few hours without losing their infectivity; the erythrocytes were washed free of the patient's serum by centrifugation at 500 × *g* for 5 min, and samples of cells suspended to a 33% hematocrit either in NHS or in the patient's serum (autologous) were freshly prepared. These suspensions were immediately presented to *A. tessellatus* mosquitoes through a water-jacketed membrane feeder at 40°C provided with a nylon filament mesh (pore size, 74 μm; Sargent-Welsh), and mosquitoes were allowed to feed for 15 to 20 min. Fed mosquitoes (from both direct and membrane feeding experiments) were maintained at 26°C and 60 to 70% relative humidity. At 6 to 7 days after feeding they were dissected, and the midguts were examined microscopically for oocysts. The mean number of oocysts per mosquito gut was used as an index of transmission.

RESULTS

A. tessellatus mosquitoes were fed directly upon 31 patients with acute infections of *P. vivax*. At the same time, blood was drawn from each patient, washed, suspended in NHS (nonmalarial), and membrane fed to mosquitoes. Autologous serum from each patient was used to measure IFT titers against air-dried female gametes of *P. vivax*.

The infectivity of patients on which mosquitoes fed directly was closely associated with the titer of antibody in their serum, as measured by IFT against air-dried gametes of *P. vivax* (Fig. 1A, Table 1). Thus, patients with titers of 1:320 or above almost invariably gave rise to mosquito infections of less than four oocysts per gut and were frequently totally noninfectious. Patients with titers of 1:160 or less almost all gave mosquito infections of at least 20 oocysts per gut and frequently much higher infectivities. The infectivity of the parasites when fed to mosquitoes in NHS had no correlation with IFT titers of antigamete antibodies (Fig. 1B, Table 1).

For 8 of these 31 patients, mosquitoes were membrane fed with the patient's blood resuspended in its own (autologous) serum. These results were compared with those from simultaneous membrane feedings in NHS and with direct feeding on the patient (Table 2). There was a strong correlation between the infectivity of a patient by direct feeding and suppression of infectivity mediated by the autologous serum in membrane feeding. It was also apparent from the results of direct feeding experiments and the membrane feedings of the parasites in autologous serum that membrane feeding in general yields a lower infectivity than direct feeding; this is possibly attributable to the loss or impairment of gametocyte viability during manipulation of blood in vitro.

It was noticed that there appeared to be an association between a patient's past history of *P. vivax* malaria and his infectivity to mosquitoes. Data from the patients were collected into three groups: group I, having no past history of malaria; group II, having a past history of malaria but no attacks within the previous 4 months; and group III, having a past history of malaria with an attack(s) within the previous 4 months (Table 1). The mean infectivity of group III, having had a previous attack within 4 months, was much reduced compared with that of the other two groups (I and II). This was evident from the results of direct feeding on patients and also from the infectivity of the patients by direct feeding relative to the infectivity of their blood when membrane fed

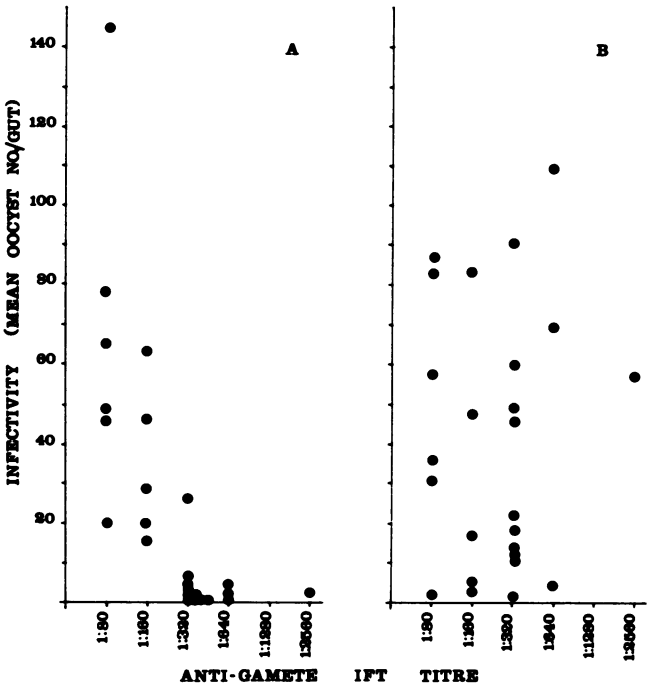

FIG. 1. Infectivity of *P. vivax* infections to *A. tessellatus* mosquitoes by direct feeding on patient (A) or by feeding patient's blood, suspended in NHS (nonmalarial), to mosquitoes through a membrane (B). Infectivity is plotted against the titer of antibodies in the patient's serum as measured by IFT against air-dried female gametes of *P. vivax*. Data for 25 patients (Table 1) are presented here.

to mosquitoes in the presence of NHS (nonmalarial) (relative infectivity). The mean titer of antigamete antibodies was approximately doubled in this group as compared with groups I and II.

Prompted by these findings, we reexamined the results of our previous study on 40 acute vivax malaria patients in Sri Lanka (7) in whom transmission-blocking immunity had been assessed by membrane feeding the patient's parasitized blood in the presence of autologous serum or NHS. In this study (7), relative infectivity was expressed as infectivity of a patient's parasitized blood fed to mosquitoes in autologous serum as a percentage of that in NHS (Table 3). Here too, those who had experienced a prior malarial attack within the previous 4 months had much higher levels of transmission-blocking immunity (and of antigamete antibodies) than the other groups.

The duration of transmission-blocking immunity following *P. vivax* infection was further studied in another group of six patients. Serum samples were collected for up to 100 days after diagnosis and cure of *P. vivax* infection and tested for transmission-blocking activity in membrane feeding experiments, and titers against air-dried *P. vivax* female gametes were determined by IFT (Fig. 2). None of these patients experienced a malaria infection during the follow-up period. In all six patients IFT titers remained elevated, and the sera strongly suppressed infectivity to mosquitoes for at least 1 month after initial diagnosis. Sera from all patients, however, lost all or some of their transmission-blocking capacity and had undergone a corresponding reduction in IFT titers within 2 to 3 months after diagnosis and cure. Serum from one patient lost its transmission-blocking ability and gave way to pronounced infectivity enhancement during this

TABLE 1. Effect of antigamete antibodies on infectivity of patients to *A. tessellatus* mosquitoes

Group[a]	Patient no.	% Gametocytemia	Attack no.	Antigamete IFT titer (reciprocal)	Oocysts/gut (no. infected/total)		Relative infectivity (%) of direct feeding[b]
					Membrane feeding in NHS	Direct feeding	
I	MR 458	0.07	1	160	5.81 (13/32)	28.09 (19/22)	483.48
	MR 470	0.10	1	640	109.50 (6/6)	0.73 (4/15)	0.67
	MR 523	0.08	1	80	86.70 (8/10)	208.42 (18/19)	240.39
	MR 540	0.14	1	320	19.36 (7/11)	0.33 (2/6)	1.70
	MR 575	0.06	1	160	17.10 (1/6)	46.66 (4/6)	272.87
	MR 584	0.21	1	320	12.20 (4/10)	4.0 (6/12)	32.79
	MR 614	0.15	1	80	83.80 (7/10)	78.3 (12/16)	93.44
	MR 524	0.05	1	160	3.29 (6/24)	15.2 (5/11)	462.01
	MR 690*[c]	0.28	1	640	63.85 (5/7)	1.0 (1/4)	1.57
	MR 722*	0.007	1	640	12.48 (11/12)	0 (0/10)	0
	MR 724	0.03	1	320	1.80 (2/5)	2.4 (2/8)	133.33
	Mean[d]	0.10		249	18.52	7.94	33.15 (1.09)[e]
II	MR 462	0.19	3	80	30.66 (16/29)	49.75 (7/8)	162.26
	MR 476	0.04	3	640	69.11 (8/9)	3.17 (4/12)	4.59
	MR 489	0.04	4	320	13.70 (15/17)	0 (0/15)	0
	MR 494	0.12	4	160	82.80 (5/5)	63.46 (13/13)	76.64
	MR 497	0.13	2	80	3.46 (3/13)	20.25 (7/8)	585.26
	MR 579	0.05	6	160	48.70 (6/10)	20.10 (9/12)	41.27
	MR 603	0.04	3	80	36.12 (16/16)	45.50 (21/22)	125.97
	MR 698*	0.02	2	320	28.60 (9/10)	81.45 (11/11)	284.79
	Mean	0.11		175	28.36	18.78	52.15 (1.11)[e]
III	MR 519	0.06	3	640	3.60 (10/19)	0 (0/15)	0
	MR 620	0.05	2	320	46.40 (8/10)	0 (0/20)	0
	MR 447	0.09	5	320	22.08 (10/12)	0.6 (6/25)	2.72
	MR 457	0.07	2	320	91.00 (4/5)	26.02 (3/4)	28.59
	MR 465	0.29	2	2,560	57.90 (15/20)	3.26 (8/15)	5.63
	MR 475	0.06	2	320	48.00 (7/9)	0.12 (2/25)	0.25
	MR 505	0.13	2	320	11.55 (9/13)	2.80 (5/13)	24.24
	MR 566	0.13	5	80	58.76 (8/14)	65.50 (16/20)	111.47
	MR 602	0.09	2	320	61.00 (7/11)	0 (0/10)	0
	MR 703*	NA[f]	5	NA	55.96 (9/10)	0.14 (1/7)	0.25
	MR 706*	0.01	4	1,280	2.90 (6/10)	0 (0/15)	0
	MR 726*	0.11	6	640	45.01 (14/15)	12.60 (2/5)	27.99
	Mean	0.10		439	28.46	2.11	3.62 (0.65)[e]

[a] Group I, No past history of malaria. Group II, Past history of malaria; previous attack occurred over 4 months ago. Group III, Past history of malaria; previous attack occurred within past 4 months.
[b] Compared with membrane feeding in NHS.
[c] *, Data for all patients except those indicated with an asterisk have been included in Fig. 1.
[d] Geometric mean.
[e] Mean ×/÷ standard error.
[f] NA, Not available.

period, producing an almost eightfold increase of infectivity compared with controls.

DISCUSSION

We had previously shown that serum from acute *P. vivax* infection in humans frequently contains antibodies which suppress infectivity of the parasites to mosquitoes (7). These studies had been done by feeding a patient's *P. vivax*-infected blood cells, combined with autologous serum (patient's own) or NHS (nonmalarial), to mosquitoes through a membrane feeding apparatus. This approach allowed a controlled comparison of the effects of a patient's own serum with those of nonmalarial serum; however, the relationship of these results to the actual infectivity of a patient to mosquitoes could only be inferred. In the present study we fed mosquitoes on *P. vivax*-infected patients in an attempt to determine the direct effect of such transmission-blocking antibodies on the infectivity of human infection to mosquitoes.

TABLE 2. Comparison of infectivity of *P. virax* infections to *A. tessellatus* mosquitoes by various feeding methods

Patient no.	Antigamete IFT titer (reciprocal)	Infectivity to mosquitoes (oocysts/gut)		
		Direct feeding	Membrane feeding in autologous serum	Membrane feeding in NHS
MR 462	80	49.75	12.53	30.66
MR 603	80	45.50	4.62	36.12
MR 458	160	28.09	18.20	5.81
MR 457	320	26.20	0.72	91.00
MR 465	2,560	3.28	0	57.90
MR 476	640	3.17	0.05	69.11
MR 447	320	0.60	0	22.08
MR 475	320	0.12	0	48.00

TABLE 3. Infectivity (assessed by membrane feeding) and transmission-blocking immune status of patients with relation to past malaria history[a]

Group	No. of patients	Mean % gametocytemia	Mean antigamete IFT titer (reciprocal)	Oocysts/gut (no. infected/total) after membrane feeding in:		Relative infectivity[b] (mean % ×/÷ SE)
				NHS	Autologous serum	
I	14	0.09	145	18.42 (139/246)	2.59 (84/244)	8.90 ×/÷ 0.69
II	13	0.11	320	12.97 (128/220)	0.41 (25/231)	1.45 ×/÷ 0.48
III	13	0.08	288	25.00 (115/181)	0.09 (11/163)	0.26 ×/÷ 0.14

[a] Forty patients from a previous study (7). Groups were as described in the text and in Table 1, footnote *a*. Values are all geometric means.
[b] Compared with membrane feeding in NHS.

The results of this study have demonstrated a clear association between infectivity of human *P. vivax* infection to mosquitoes and the presence of transmission-blocking factors in the serum of the infected individual. The effects, moreover, correlated closely with the level of serum antibodies to air-dried *P. vivax* gametes as measured by IFT. Patients with titers of less than 1:160 were generally highly infectious to mosquitoes; those with titers of 1:320 and above were poorly infectious or did not infect at all. In the few patients in whom the transmission-blocking ability of the sera was assessed in membrane feeding experiments, it was confirmed that the absolute infectivity of the patients to mosquitoes correlated with the transmission-blocking ability of their sera.

As we have previously reported, antigamete antibodies at low concentrations enhance rather than block infectivity of the parasite (8; J. S. M. Peiris, S. Premawansa, P. V. Udagama, M. B. R. Ranakawa, M. U. Nanayakkara, Y. D. Munesinghe, C. P. Gamage, P. H. David, R. Carter, and K. N. Mendis, Am. J. Trop. Med. Hyg., in press); some acute-phase sera with low antibody titers (e.g., MR 458 in Table 2), as well as a convalescent-phase serum, showed pronounced infectivity-enhancing effects.

The antigens accessible to antibody in the IFT assay against air-dried gametes include internal antigens as well as those that would be exposed on the gamete surface; the assay is, therefore, probably measuring antibodies against antigens in both types of location. Presumably antibodies against the gamete surface antigens, which are likely to include those which mediate transmission-blocking immunity, and antibodies against internal antigens are induced to a similar degree during *P. vivax* infection and account for the close parallel between effective suppression of infectivity and IFT titer against the air-dried gametes. The sharp transition from titers associated with high and low infectivity is, nevertheless, striking. Similar sharp transitions have, however, been described for titers of antigamete surface antibodies in correlation with transmission blocking in animal malaria systems (1).

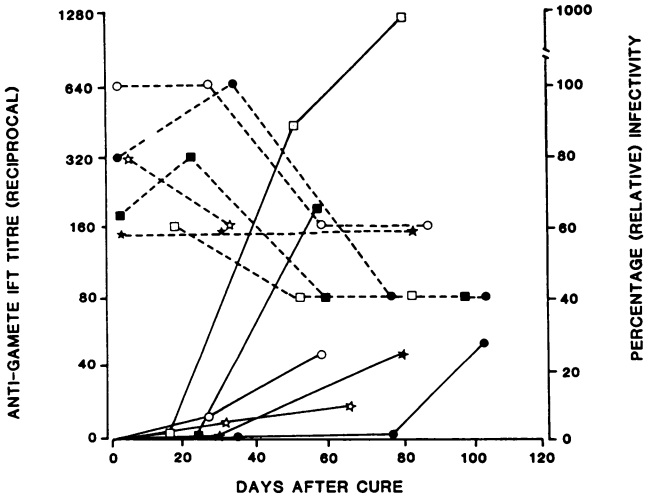

FIG. 2. Antigamete antibody levels (by IFT against air-dried gametes) (– – –) and infectivity-suppressive effects (——) in the sera of six vivax malaria patients during acute infection and convalescence.

In our previous study (7) we found a strong association between previous malaria attacks and increased suppression of infectivity by serum from *P. vivax*-infected patients. As a group, sera from patients who had experienced at least one previous attack suppressed infectivity 5 to 10 times more than sera from patients experiencing their first attack of *P. vivax*. In the present study we have found that this boosting effect is mainly due to attacks within less than 4 months before a current *P. vivax* infection. Beyond this time, the influence of previous malarial episodes on the infectivity of a current infection is much reduced. This short-term boosting of transmission-blocking immunity was associated with a similar short-term boosting of antigamete antibodies as measured by IFT.

Boosting of transmission-blocking immunity has been shown to accompany homologous challenge with active blood infections up to at least 6 months after gamete immunization in chickens (1) and mice (6) and to be maintained for at least 3 years with intermittent challenge infections in rhesus monkeys (5). In human infections with *P. vivax*, our results imply a rather inefficient "memory" for transmission-blocking immunity. It is possible that much of the effective boosting of transmission-blocking immunity following a recent malarial attack is no more than the result of new transmission-blocking antibodies, induced by a current attack, added to the levels of those persisting from the previous one. This would be approximately consistent with the duration of effective levels of such antibodies demonstrated after a *P. vivax* attack in the present study (Fig. 2). Our results do not suggest that there is a very efficient response by memory helper T-cells interacting with previously encountered immunogens.

Whatever the mechanism accounting for the relatively short interval within which transmission-blocking immunity may be boosted by successive *P. vivax* infections, the epidemiological consequences are of great interest. Thus, a situation in which a succession of relatively frequent attacks of *P. vivax* leads to increasingly effective transmission-blocking immunity could apply a significant curb on a rising seasonal epidemic of *P. vivax* malaria. On the other hand, the failure of successive attacks of *P. vivax* to boost when

separated by intervals of an interseasonal length (e.g., greater than 4 months) not only would ensure that transmission-blocking immunity from the previous season does not compromise the onset of the following season's *P. vivax* transmission, but also, by leading to low levels of antibodies, would even enhance infectivity. These concepts are now being tested in a field study on seasonal *P. vivax* malaria epidemics in Sri Lanka.

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