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Antibodies to *Anopheles* midgut reduce vector competence for *Plasmodium vivax* malaria

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Abstract. *Anopheles tessellatus* mosquitoes ingested *Plasmodium vivax* gametocytes in human erythrocytes suspended in rabbit sera with and without anti-mosquito midgut antibodies. When the mosquito bloodmeal contained anti-midgut antibodies, fewer oocysts of *P.vivax* developed on the mosquito midgut and the proportion of mosquitoes becoming infected was significantly reduced. Complement inactivated serum also reduced the infection rate and load. A second bloodmeal containing anti-midgut antibodies, given 48 or 72 h later, did not enhance the transmission-blocking effect. IgG purified from anti-midgut sera was shown to mediate the transmission-blocking effect.

Key words. *Anopheles tessellatus*, *Plasmodium vivax*, anti-mosquito antibodies, malaria, mosquito midgut, transmission blocking immunity.

Introduction

Male and female gametocytes of malaria parasites, *Plasmodium* spp., infect vector mosquitoes when ingested in a bloodmeal. Gamete formation and fertilization occur in the lumen of the mosquito midgut. The resulting motile ookinetes traverse the single layer of epithelial cells of the midgut and lodge beneath the basal lamina, on the haemocoel side, to become oocysts. Sporozoites produced within oocysts migrate to the salivary glands in order to infect a vertebrate host when the mosquito bites.

The development of malaria parasites within their mosquito vector is closely linked to mosquito physiology. For example, exflagellation of the male gametocyte of the avian malaria parasite *P.gallinaceum* Brumpt is stimulated by a mosquito factor (Nijhout, 1979); the level of trypsin in the midgut of *Aedes aegypti* L. influences the number of *P.gallinaceum* oocysts produced (Gass, 1979) and the production of chitinase in *P.gallinaceum* ookinetes is linked to the need to digest the peritrophic membrane formed around a bloodmeal (Huber *et al.*, 1991). It has been postulated that an epithelial cell receptor for ookinetes facilitates their movement across the midgut (Ramasamy & Ramasamy, 1990). Surface receptors in specific areas of the salivary glands of *Ae.aegypti* have also been reported to govern the invasion of *P.gallinaceum* sporozoites to particular lobes of the glands (Perrone *et al.*, 1986).

Cellular and humoral responses to arthropod vector antigens also modulate the transmission of pathogens. For example, ingestion of anti-mosquito sera in an infective bloodmeal inhibits the infectivity of arboviruses (Ramasamy *et al.*, 1990) and the murine malaria parasite *P.berghei* Vincke (Ramasamy & Ramasamy, 1990; Schriefer *et al.*, 1993) to vector mosquitoes. Ingestion of anti-mosquito antibodies also reduced the longevity (Alger & Cabrera, 1972), and the fecundity of anopheline mosquitoes (Ramasamy *et al.*, 1992; Srikrishnaraj *et al.*, 1993).

In preliminary experiments with the human malaria parasite *Plasmodium vivax* Grassi human malaria and the mosquito *Anopheles tessellatus* Theobald, a natural vector in Sri Lanka (Mendis *et al.*, 1990), we found that fewer oocysts developed when the mosquitoes ingested infective *P.vivax* gametocytes in a bloodmeal carrying mosquito antisera (Ramasamy *et al.*, 1993). We show here that ingestion of IgG antibodies to the mosquito midgut reduces the infectivity of *P.vivax* to *An.tessellatus* in a complement independent manner.

Materials and Methods

Mosquito colony and antisera. A colony of *An.tessellatus* was maintained in the laboratory at 28 ± 1°C and 80% r.h. (Ramasamy *et al.*, 1992). Polyclonal antibodies to midgut

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antigens were produced by immunizing New Zealand white rabbits with midgut tissue derived from 4–7-day-old sugar-fed *An.tessellatus* females, while control rabbits were injected with phosphate-buffered saline (PBS), pH 7.2, the first immunizations were emulsified in Freund's complete adjuvant (Ramasamy *et al.*, 1992). Antibody titres in immune sera and purified IgG (used at 1.8–2.1 mg/ml starting protein concentration) were measured with an enzyme-linked immunosorbent assay (ELISA) against midgut tissue (Ramasamy *et al.*, 1992). Sera from two or three non-immunized age-matched rabbits were used for infectivity studies.

IgG antibodies. Immune rabbit sera were precipitated with 50% saturated ammonium sulphate at 4°C. The precipitated protein was dissolved in 5 mM sodium phosphate, pH 6.5, and dialysed overnight against two changes of 5 mM sodium phosphate. Contaminating serum proteins were removed by binding to DEAE cellulose in 5 mM sodium phosphate (pH 6.5) and purity of the IgG was confirmed by separating an aliquot by sodium dodecyl sulphate–polyacrylamide gel electrophoresis, SDS-PAGE (Laemmli, 1970). Only gamma heavy and light chains were observed in the stained gel. The purified IgG was then extensively dialysed against PBS at 4°C and aliquots stored at −20°C for use in experiments.

Infectivity studies. P.vivax-infected blood samples were obtained voluntarily from patients seeking treatment at the Government Hospital, Rambukkana, where malaria is endemic. Parasitaemia and gametocytaemia were determined by examining Giemsa-stained bloodsmears. 5–10 ml of venous blood was drawn from each patient and diluted immediately in 10 volumes of suspended animation (SA) solution (10 mM Tris, 170 mM NaCl, 10 mM glucose, pH 7.4) which reversibly suppressed gametogenesis (Carter & Nijhout, 1977; Munasinghe *et al.*, 1983). Single or pooled patient blood samples diluted in SA solution were centrifuged at 500*g* for 10 min at room temperature. The plasma was removed and 300 µl of parasitized human erythrocytes were mixed with 225 µl non-immune rabbit serum and 75 µl of rabbit immune serum or IgG. Reconstituted blood was fed, via a membrane of stretched parafilm, to non-bloodfed 3–4-day-old *An.tessellatus*, using a water-jacketed glass feeder at 40–41°C. Mosquitoes were permitted to feed for 15–20 min and those fully engorged with blood (*c.* 30%) were separated and maintained in the insectary on 10% glucose and multivitamins. Mosquito mortality was counted daily. Surviving mosquitoes were dissected 8–10 days after the infective blood-feed. Their midgut was examined and viable oocysts counted (using methylene blue staining) as a measure of *An.tessellatus* susceptibility to *P.vivax*.

To test the effect of serum complement on the susceptibility of *An.tessellatus* to *P.vivax*, 300 µl parasitized human erythrocytes were mixed with 75 µl of IgG antibodies and 225 µl of either fresh or heat-inactivated serum prepared from the same non-immunized rabbit, offered to mosquitoes through a membrane feeder.

To test the effect of multiple bloodmeals containing anti-mosquito antibodies on vector susceptibility to parasite infection, a second bloodmeal containing immune rabbit serum mixed with non-parasitized human erythrocytes (in equal proportions by volume) was given either 48 or 72 h after the first infective bloodmeal containing immune rabbit serum. Mosquitoes were dissected 8–10 days after the first infective bloodmeal.

Statistical analysis. Chi-square tests were used to assess the significance of differences between the proportions of infected mosquitoes resulting from feeds on different sera and IgG preparations, as well as differences in the mortality-rates of mosquitoes fed on different sera and antibodies. The Mann-Whitney modification of the Wilcoxon sum of ranks test was used to analyse differences in oocyst numbers between mosquitoes in each experiment. For logistical reasons (e.g. the amount infected blood withdrawn from a patient) the numbers of mosquitoes used in an experiment could not be further increased. Hence, for statistical purposes, the infection rates in mosquitoes fed on different anti-midgut antibody preparations were pooled for comparision with infection rates in control mosquitoes.

Results

The presence of rabbit antisera raised against mosquito midgut antigen, in a bloodmeal reduced the susceptibility

Table 1. Effect of anti-midgut sera on susceptibility of *An.tessellatus* to *P.vivax*.

Rabbit	Immunizing antigen	Antibody titre*	Mortality (%) 48 h (*n*)	Oocyst range (median)	Infection (%)† (*n*)	
9	Midgut	10^{10}	19.2 (156)	1–3 (2)	7.8 (90)	I
10	Midgut	10^{9}	29.3 (157)	1–12 (2–3)	21.9 (82)	
11	Midgut	10^{8}	25.0 (112)	1–4 (2)	14.3 (56)	$\chi^2 = 23.7$ $P < 0.001$
12	Midgut	10^{9}	24.3 (74)	1–17 (5)	31.1 (45)	
13	PBS	0	22.4 (129)	1–20 (6)	42.0 (81)	II
14	PBS	0	22.9 (83)	1–21 (7)	35.4 (48)	

* Antibody titres were determined by ELISA.

† The proportions of infected mosquitoes obtained by feeding on anti-midgut sera (rabbits 9–12 group I) were pooled and compared by the chi-square test with mosquitoes fed on control sera (rabbits 13 and 14, group II). Gametocytaemia in the bloodmeal was 0.02%. *n* = number of mosquitoes.

Table 2. Effect of IgG anti-midgut antibodies on the susceptibility of *An.tessellatus* to *P.vivax*.

Rabbit	Immunizing antigen	Antibody titre*	Mortality (%) 48 h (*n*)	Oocyst range (median)	Infection (%)† (*n*)		
Experiment 1							
9	Midgut	10^4	10.2 (88)	2–12 (5)	9.6 (73)	I	$\tau^2 = 3.6$; $0.05 < P < 0.1$
11		10^4	9.5 (84)	1–7 (1)	13.0 (69)		
13	PBS	0	15.4 (78)	1–12 (3.5)	17.2 (58)	II	
14		0	16.9 (65)	1–12 (4)	19.6 (46)		
Experiment 2							
9	Midgut	10^4	7.1 (113)	1–16 (5.5)	19.1 (94)	I	$\chi^2 = 39.1$; $P < 0.001$
11		10^4	6.0 (116)	2–24 (8)	15.5 (97)		
13	PBS	0	8.7 (115)	1–19 (5)	49.5 (91)	II	
14		0	7.5 (107)	1–15 (4.5)	45.2 (84)		
Experiment 3							
9	Midgut	10^4	16.2 (97)	1–18 (6)	26.7 (45)	I	$\chi^2 = 11.5$; $P < 0.001$
11		10^4	9.7 (72)	2–14 (4)	38.9 (36)		
13	PBS	0	13.4 (97)	2–23 (9)	55.6 (45)	II	
14		0	12.4 (81)	2–17 (8)	60.9 (41)		

IgG from rabbits was used at 0.5 mg/ml in the serum of the bloodmeal.

* Antibody titres were determined by ELISA.

† The proportions of infected mosquitoes obtained by feeding on IgG from rabbits 9 and 11 (group I) were pooled and compared by the chi-square test with mosquitoes fed IgG from rabbits 13 and 14 (group II). The effects of IgG of rabbits immunized with the same antigen (i.e. nos. 9 and 11; 13 and 14) were not significantly different. Gametocytaemia in experiments 1, 2 and 3 was 0.03%, 0.01% and 0.03%, respectively. *n* = number of mosquitoes.

of *An.tessellatus* to *P.vivax* (Table 1). When infection rates in mosquitoes fed on the four different anti-midgut sera were pooled, and compared to the infection rates in mosquitoes fed on the two control rabbit sera, the reduction in infectivity was statistically significant ($\chi^2 = 23.7$; $P < 0.001$). However, differences in infectivity were observed between the four anti-midgut sera. The immune sera from rabbits 9 and 11 were significantly more inhibitory; the immune serum from rabbit 9 produced significantly fewer infections than immune sera from either rabbit 10 ($\chi^2 = 6.9$; $P < 0.01$) or rabbit 12 ($\chi^2 = 12.4$; $P < 0.001$).

Table 3. Effect of complement on the susceptibility of *An. tessellatus* to *P.vivax*.

Rabbit	Immunizing antigen	Non-immune rabbit serum	Infection (%) (*n*)
11	Midgut	Non-heat inactivated	56.3 (64)[a]
		Heat inactivated	62.3 (61)[b]
13	PBS	Non-heat inactivated	78.8 (52)[a]
		Heat inactivated	84.5 (58)[b]

IgG from rabbit 11 was used at 0.5 mg/ml in the serum of the bloodmeal. The results of percentage infections indicated with superscript *a* are significantly different ($\chi^2 = 6.6$; $P < 0.05$) as are those indicated by superscript *b* ($\chi^2 = 7.5$, $P < 0.01$). Gametocytaemia in the bloodmeal was 0.08%. *n* = number of mosquitoes.

Similarly, the immune serum from rabbit 11 was significantly more inhibitory than the serum from rabbit 12 ($\chi^2 = 4.2$; $P < 0.05$). Mosquitoes fed on sera from the two control rabbits did not differ significantly in their infection rates. Significant differences between mosquitoes fed on immune (anti-midgut) and control sera were not seen in the 48 h mortality rates. However, the numbers of oocysts per mosquito were fewer in those fed on immune sera when compared to mosquitoes fed control sera ($Z = 4.4$; $P < 0.01$).

Results similar to those shown in Table 1 were observed when 50% ammonium sulphate precipitated fraction of sera was fed to mosquitoes (data not shown). When used for *in vitro* feeding, IgG prepared from the sera of the two immunized rabbits that produced the greatest inhibitory activity (viz rabbits 9 and 11) showed a reduction in the susceptibility of *An.tessellatus* to *P.vivax*. These reductions were compared to that of control feeds containing IgG from rabbits 13 and 14; the results were statistically significant in two out of three experiments (Table 2). The IgG antibodies had no effect on the 48 h mortality of the mosquitoes. The arithmetic mean numbers of oocysts per mosquito were fewer in those fed anti-midgut IgG in all three experiments, but significantly so ($Z = 2.28$; $P < 0.05$) only in experiment 3 (Table 2). The presence of complement in the infective bloodmeal did not have a significant effect on parasite infectivity (Table 3). Both heat-inactivated and fresh rabbit serum, to which purified anti-midgut

Table 4. Effect of varying the concentration of IgG anti-midgut antibodies on the susceptibility of *An.tessellatus* to *P.vivax*.

Rabbit	Immunizing antigen	Concentration of IgG (mg/ml)	Oocyst range	Infection (%) (*n*)	Reduction in infection (%)
11	Midgut	0.5	3–70	72.5 (69)[a]	23
11	Midgut	0.05	8–77	86.4 (59)	8
11	Midgut	0.005	5–74	82.5 (57)	12
13	PBS	0.5	2–90	93.6 (78)[a]	–

The concentration of IgG given refers to total IgG in the bloodmeal. The percentage of infections indicated by the superscript *a* are significantly different ($\chi^2 = 11.9$; $P < 0.001$). Gametocytaemia in the bloodmeal was 0.01%. *n* = number of mosquitoes.

Table 5. Effect of two bloodmeals containing anti-mosquito antibodies on the susceptibility of *An.tessellatus* to *P.vivax*.

Rabbit	Immunizing antigen	No. of blood meals	Oocyst range	Infection (%) (*n*)
Experiment 1				
9	Midgut	1	9–76	55.5 (45)
		2	4–43	53.1 (32)
13	PBS	1	4–94	74.1 (23)
		2	5–33	66.7 (24)
Experiment 2				
9	Midgut	1	1–23	37.9 (103)
		2	1–20	32.5 (83)
13	PBS	1	2–32	54.8 (62)
		2	2–20	48.0 (48)

The second bloodmeal was given 72 h after the first bloodmeal in experiment 1 and 48 h after the first bloodmeal in experiment 2. *n* = no. of mosquitoes.

IgG was added in the infective blood-feed, produced statistically significant reductions in infectivity: 26.3% and 28.6% respectively, compared to the control.

The effect of diluting IgG antibodies from one rabbit (no. 11) on the infectivity of *P.vivax* is shown in Table 4. A second bloodmeal of non-parasitized human erythrocytes and immune serum, given either 48 or 72 h after the first infective feed containing the same immune serum, did not enhance the reduction in parasite infection (Table 5).

Discussion

Preliminary investigations showed that rabbit antisera to *An.tessellatus* tissues (head – thorax, midgut, rest of the abdomen) reduced the susceptibility of this vector to *P.vivax* (Ramasamy *et al.*, 1993). In those experiments, the rabbit antisera were heat inactivated at 56°C for 30 min and then absorbed with washed human erythrocytes to remove rabbit antibodies reacting with human cells. The absorbed rabbit sera were then mixed with fresh human serum (of the same blood group as the gametocyte carrying blood) for feeding to mosquitoes. In the present series of experiments, when unabsorbed rabbit sera or IgG were directly mixed with human erythrocytes before feeding, a similar inhibition of parasite transmission was seen. Significant agglutination of human erythrocytes by natural rabbit antibodies, within the feeding period, was not observed in the present investigation.

Inhibition of transmission by IgG, purified from rabbit anti-midgut sera, confirms that the active component in immune sera are antibody molecules. Immune sera contain antibodies with specificities against different midgut molecules. It is possible that while some antibodies (e.g. against epithelial cell surfaces) block transmission, others (e.g. against peritrophic membrane) enhance transmission so that only the net effect on transmission of the parasite to mosquitoes is observed in the experimental system. The use of monoclonal anti-midgut antibodies should help to elucidate the mechanisms involved and such work is currently underway.

Because immune IgG was effective only at high concentrations, it is possible that only a small proportion of the antibodies in immune sera have the specificities required to block parasite transmission. Our results show that complement is not required for the action of anti-mosquito antibodies. Therefore the effects are not produced by complement mediated lysis of midgut epithelial cells. The absence of damage to the epithelium has been confirmed by electron microscopy of the midguts of mosquitoes fed on immune sera. In contrast, a role for complement was indicated in antibody mediated damage to the gut of the cattle tick, *Boophilus microplus* Canestrini (Kemp *et al.*, 1986).

Failure to enhance the transmission reducing effect with a second bloodmeal containing rabbit anti-midgut sera suggests that the antibodies exert their effect only during the period of gamete formation, fertilization and ookinete penetration of the mosquito gut. With *P.vivax* these processes occur within 24 h of the entry of gametocytes into the midgut of the vector mosquito. A significant reduction in the number of *P.vivax* oocysts per midgut, in the presence of anti-midgut antibodies, was observed in some experiments in the present investigation. This result is consistent with the model proposed for the *P.berghei*/*An.farauti* Laveran system (Ramasamy & Ramasamy,

1990), where antibodies were postulated to function by blocking the passage of ookinetes through the midgut.

Antisera used in this investigation reduce fecundity in *An.tessellatus* (Ramasamy *et al.*, 1992) and have been used to characterize antigens in mosquito tissues (Ramasamy *et al.*, 1991). The target molecules for the fecundity reducing effect, and their relationship to the molecules mediating a reduction in the infectivity of malaria parasites, are yet to be determined. Identifying such molecules, and others which confer refractoriness to malaria parasites in anopheline vectors (Collins *et al.*, 1986), would be useful for developing new methods of controlling malaria.

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