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Effect of anti-mosquito antibodies on the infectivity of the rodent malaria parasite *Plasmodium berghei* to *Anopheles farauti*

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ABSTRACT. The effect of mouse anti-mosquito antibodies, present in the bloodmeal, on the infectivity of *Plasmodium berghei* Vincke to *Anopheles farauti* Laveran was investigated. Significantly fewer oocysts developed in mosquitoes feeding on mice immunized with sugar-fed mosquito midgut antigens than in mosquitoes feeding on control mice. Mosquitoes feeding on mice immunized with the midgut antigens derived from sugar-fed mosquitoes also showed reduced mortality and had lower infection rates than those fed on unimmunized mice. Blood-fed midgut antigen was less effective in producing these effects than sugar-fed midgut antigen.

Key words. *Anopheles farauti,* anti-mosquito antibodies, midgut antigens, oocysts, *Plasmodium berghei,* transmission blocking, vector–parasite interactions.

Introduction

Male and female gametocytes of the malaria parasite, *Plasmodium,* are infectious to the mosquito vector when ingested in the bloodmeal. After fertilization in the lumen of the mosquito midgut, the zygote develops into a motile ookinete that traverses the midgut epithelium, to become an oocyst on the haemocoel side of the midgut. Sporozoites are produced within the oocyst and then migrate to the salivary glands, where they become able to infect another vertebrate host when the mosquito takes its next bloodmeal. The maturation of the sexual stages of *Plasmodium* and the transmission of sporozoites to vertebrates is closely dependent on the physiology of the mosquito. For example, exflagellation of the male gametocyte of the avian malaria parasite *P.gallinaceum* Brumpt is stimulated by a mosquito factor (Nijhout, 1979) and the level of trypsin in the midgut of *Aedes aegypti* L. influences the number of *P.gallinaceum* oocysts produced (Gass, 1977; Gass & Yeates, 1979). 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).

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Host antibodies to mosquito tissues affect mosquito physiology (Alger & Cabrera, 1972; Sutherland & Ewen, 1974; Ramasamy *et al.*, 1988a, b), and this may be at least in part due to antibodies or their fragments traversing the mosquito midgut and affecting target tissues (Ramasamy *et al.*, 1988a). Antibodies against midgut components of *Ae.aegypti,* when ingested with an infected bloodmeal, have recently been shown to reduce the susceptibility of the mosquito to infection with arboviruses (Ramasamy *et al.*, 1990). This effect might, in certain instances, be exploited for the control of the transmission of arboviral diseases to vertebrates.

Anopheles farauti Laveran is a vector of the human malaria parasites *P.falciparum* Welch and *P.vivax* Grassi in the Pacific region. *An.farauti* is also a laboratory vector of the rodent malaria parasite *P.berghei* Vincke

(Schuurkamp, 1982). It has been shown that host antibodies to surface antigens on gametes, taken in with an infective bloodmeal, block the infectivity of *P.gallinaceum* to mosquitoes (Huff *et al.*, 1958; Gwadz, 1976). This phenomenon, termed transmission blocking immunity, has been also reported to occur in *P.falciparum* (Rener *et al.*, 1983) and *P.vivax* (Munesinghe *et al.*, 1986) when infecting anopheline mosquitoes. Antibodies to a surface antigen on the zygote and ookinete also block transmission (Vermeulen *et al.*, 1985). These observations are the basis for the development of a transmission-blocking vaccine against human malaria, which is being actively pursued in a number of laboratories. However, depending on the concentration of antibodies, enhancement of transmission is sometimes seen (Peiris *et al.*, 1988) and this may constitute a serious drawback to the use of antigens of sexual stages for producing transmission-blocking immunity. It has also been reported that ingested anti-sporozoite antibodies cross the mosquito midgut of *P. falciparum* infected mosquitoes, bind to antigen present on the oocysts and enhance sporozoite production (Vaughan *et al.*, 1988). We have investigated the effects of host anti-mosquito antibodies present in the infective bloodmeal on the transmission of *P.berghei* to *An.farauti*. The results show that immunization with the appropriate mosquito antigens may constitute a novel method of reducing the transmission of malaria parasites to mosquitoes.

Materials and Methods

Mosquitoes. A.farauti larvae were maintained on a diet of powdered fish-food flakes and adults fed on 10% sucrose supplemented with multivitamins. Adults were held at 27±1°C, 70–80% r.h. and LD 13:11 photoperiod. For blood-feeding, 3–7-day old female adults of *An.farauti* were starved for 24 h and fed until engorged on restrained mice.

Antigen preparation and immunizations. 3–7-day-old sugar-fed or blood-fed *An.farauti* females were killed 24 h post-engorgement and held at −20°C until dissected. The midguts of sugar-fed mosquitoes and the midgut and head/thorax of blood-fed mosquitoes were dissected separately and used as three antigen preparations. Mosquito tissue was homogenized in 0.01 M phosphate buffered saline, pH 7.2 (PBS) and suspensions injected intra-muscularly into 6-week-old BALB/C mice in a final volume of 200 μl, containing antigen derived from approximately ten mosquitoes, per mouse. The first and second immunizations were made with Freund's complete and incomplete adjuvant respectively using a 1:1 v/v emulsion of antigen and adjuvant. Subsequent immunizations were performed by intraperitoneal injection with antigen in PBS. Control mice injected simultaneously with PBS/adjuvant and PBS alone, were termed SC and BC. Groups of ten mice were immunized with sugar-fed midgut antigen (SMG); eight mice with blood-fed midgut antigen (BMG) and eight mice with blood-fed head/thorax antigen (BHT). Injections were performed at intervals of 2–4 weeks. Mice were bled from the tail and serum antibody titres to mosquito antigen determined by an enzyme-linked immunosorbant assay (ELISA) using alkaline phosphate conjugated rabbit-anti mouse IgG (Amersham, U.K.) as previously described (Ramasamy *et al.*, 1988b).

Parasitological studies. Cryopreserved rat blood from a sporozoite transmitted infection of the ANKA strain of *P.berghei* (kindly provided by the Army Malaria Unit, Sydney), was diluted 1:1 with 3.8% sterile sodium citrate and 200 μl of the mixture injected intraperitoneally into unimmunized BALB/C mice. *P.berghei* infected blood from the first rat–mouse passage was diluted in sodium citrate and 200 μl injected intraperitoneally into immunized and the corresponding control mice which had been boosted with antigen or PBS 10–12 days previously, so that each mouse received approximately 5×10^8 parasitized red cells. Thin blood films were made daily from all infected mice, stained with Giemsa and the parasitaemia and gametocytaemia determined. On day 3 after infection with *P.berghei*, immunized and control mice were restrained on corkboards and used for mosquito feeding at the same time. Individual mice were placed in netted cages with starved 3–7-day-old *An.farauti* and feeding carried out in a 21°C cabinet for 1–2 h. Blood-fed mosquitoes were held at 21°C and mortality recorded daily. Midguts of dead blood-fed mosquitoes were smeared and stained with Giemsa for detection of ookinetes. On days 10–11, blood-fed mosquitoes were dissected and the midguts examined for the presence of oocysts.

Statistical analysis. Analysis of the significance of the difference between the proportions of

dead or infected mosquitoes resulting from feeds on the immunized and control mice was performed by the chi-square test. Significant differences in the numbers of oocysts found in mosquitoes fed on immunized and control mice were determined by the Mann-Whitney modification of the Wilcoxon sum of ranks test.

Results

Mice immunized with mosquito antigen developed high ($\geq 10^4$) titres of antibodies to the immunizing antigen. Control mice, immunized with adjuvant and PBS, showed marginal antibody levels ($\leq 10^2$). The mice immunized with mosquito antigen were not protected against infection with *P.berghei*. Of the seventeen immunized mice used in infection studies, only one failed to develop parasitaemia. Both control and immunized mice achieved similar parasitaemias at the same time. The infected mice were also monitored after being used for mosquito feeding. The total parasitaemia went up to 50% and none of the mice recovered from the infection.

In a given experiment, the gametocytaemias in the mice in the immunized and control groups used for mosquito feeding were broadly within the same range (Tables 1 and 2). A high mortality was observed in *An.farauti* mosquitoes fed on the *P.berghei* infected mice. Most of the deaths occurred 24–48 h after the bloodmeal. There was no detectable correlation between the gametocytaemia in the bloodmeal and mortality. Ookinetes were observed in at least 80% of dead mosquitoes from feeds on control mice. However, it was not possible to obtain a quantitative comparison of ookinete numbers between mosquitoes fed on immunized and control mice.

In two experiments, designed to test the effect of immunization with sugar-fed midgut (SMG) antigen, 74% and 65% mean mortality rates were observed in mosquitoes fed on SC control mice (Table 1). However, the mean mortality rates in the mosquitoes fed on the corresponding immunized mice were only 16% and 48% respectively. The decrease in mortality rates were highly significant ($\chi^2=22.9$ and 16.2 respectively; $P<0.001$). The presence of the anti-SMG antibodies in the bloodmeal also significantly reduced the infectivity of the parasites. In experiment 1, mosquitoes fed on SC mice showed a mean infection rate of 60% compared to 3.4% in SMG immunized mice, a significant reduction if the data from each set of mice are pooled for comparison ($\chi^2=34.5$; $P<0.001$). The contrast would be even greater if one control mouse (D3) had not failed to infect mosquitoes. Similarly in

TABLE 1. Infectivity of *P.berghei* to *An.farauti* in the presence of antibodies to midgut components of sugar-fed mosquitoes.

Immunizing antigen	Mouse no.	Antibody titre to immunizing antigen	Gameto-cytaemia at mosquito feed	% Mortality of blood-feds*	% Infected mosquitoes†	Oocysts/gut median (range observed)
Experiment 1						
Sugar-fed midgut (SMG)	B1	10^5	0.2	0 (9)‡	0 (9)	0
	B4	10^5	0.1	32 (22)	7 (15)	4 (4)
Control (SC)	D1	10^2	0.1	52 (27)	93 (14)	75 (5–160)
	D2	10^2	0.1	79 (28)	86 (7)	100 (51–155)
	D3	10^2	0.1	91 (21)	0 (2)	0
Experiment 2						
Sugar-fed midgut (SMG)	A1	10^5	0.5	42 (26)	60 (15)	6 (1–56)
	A4	10^5	0.9	38 (99)	63 (60)	4.5 (1–42)
	C1	10^5	0.5	60 (30)	50 (12)	8 (3–18)
	C4	10^5	1.2	50 (26)	31 (13)	1.5 (1–7)
Control (SC)	E2	10^1	0.3	47 (19)	80 (10)	47 (22–102)
	E3	10^1	0.3	73 (56)	86 (14)	38 (14–132)
	E4	10^1	0.2	73 (30)	88 (8)	129 (8–163)

* Total up to 10–11 days after bloodmeal. † On day 10–11 after bloodmeal. ‡ No. of observations are given in parentheses.

TABLE 2. Infectivity of *P.berghei* to *An.farauti* in the presence of antibodies to head/thorax and midgut components of blood-fed mosquitoes.

Immunizing antigen	Mouse no.	Antibody titre to immunizing antigen	Gameto-cytaemia at mosquito feed	% Mortality of blood-feds*	% Infected mosquitoes†	Oocysts/gut median (range observed)
Experiment 1						
Blood-fed	A1	10^6	0.8	100 (10)‡	–	–
head/thorax	A2	10^6	0.5	63 (19)	43 (7)	28 (27–55)
(BHT)						
Blood-fed	C1	10^4	0.6	92 (13)	0 (1)	0
midgut	C4	10^4	0.2	23 (47)	81 (36)	43 (3–253)
(BMG)	C4a	10^4	0.1	17 (30)	8 (25)	3.5 (1–6)
Control	F1	10^1	0.2	58 (38)	63 (16)	7.5 (3–30)
(BC)	F2	10^1	0.8	84 (19)	67 (3)	39 (7–71)
	F3	10^1	1.5	95 (21)	100 (1)	8 (8)
Experiment 2						
Blood-fed	$B_1$3	10^6	1.0	83 (18)	67 (3)	29.5 (16–43)
head/thorax	$B_1$4	10^6	2.5	47 (30)	44 (16)	5 (1–18)
(BHT)	$B_1$4a	10^6	1.2	68 (37)	71 (14)	21.5 (4–96)
	$B_2$1	10^6	1.3	17 (12)	90 (10)	35 (12–58)
	$B_2$2	10^6	1.0	63 (30)	27 (11)	47 (6–49)
Control	F4	10^1	0.5	100 (27)	–	–
(BC)	F4a§	10^1	0.7	77 (44)	70 (10)	121 (17–145)
	F4a§	10^1	2.3	57 (35)	80 (15)	25 (1–85)

* Total up to 10–11 days after bloodmeal. † On day 10–11 after bloodmeal. ‡ No. of observations are given in parentheses. § Mouse used on consecutive days for mosquito feed.

experiment 2, the corresponding mean infection rates were 84% and 51% (χ^2=8.6; P<0.005). There were significant reductions in the numbers of oocysts per midgut in mosquitoes fed on SMG immunized mice in experiment 1 (z=2.28; P<0.05) and experiment 2 (z=6.64; P<0.001) compared to mosquitoes that fed on control mice.

The results of feeding *An.farauti* on *P.berghei* infected mice immunized with blood-fed midgut antigen (BMG) and head/thorax antigen (BHT) are presented in Table 2. Reductions in the mean mortality rates were apparently significant with mosquitoes fed on BMG immunized mice in experiment 1 (χ^2=32.4; P<0.001)) and with BHT immunized mice in experiment 2 (χ^2=8.01; P<0.005), but not in experiment 1 (χ^2=0.03; P> 0.75), in comparison to mosquitoes fed on BC control mice. Although there was a reduction in mean infection rates of mosquitoes fed on BMG immunized mice (30%) compared to controls (76%) in experiment 1, the reduction was insignificant (χ^2=1.39; P>0.10). Similarly no significant changes in the infection rates were observed in mosquitoes fed on BHT immunized mice in the two experiments (χ^2=1.04; P>0.25 and χ^2=2.65; P>0.10) respectively. Also the oocyst numbers were not significantly reduced in mosquitoes feeding on BMG and BHT immunized mice compared to BC control mice.

Discussion

Ookinetes of *P.berghei* and *P.berghei nigeriensis* Killick-Kendrick are believed to damage the midgut epithelium of the vector *An.stephensi* Liston, possibly because of their intracellular mode of penetration of the gut (Canning & Sinden, 1973; Davies, 1974), and this has been reported to cause mosquito mortality (Gad *et al.*, 1979; Becker-Feldmann *et al.*, 1985). Malaria infected mosquitoes also show other pathological changes (Maier *et al.*, 1987). However, the penetration of *P.falciparum* through the midgut of *An.stephensi* may occur through an intercellular pathway (Meis & Ponnadurai, 1987a) and apparently does not cause high mortality in heavily infected *An.stephensi* and *An.gambiae* Giles (Meis & Ponnadurai, 1987b). In contrast, we

have observed high mortality in *An.farauti* infected with *P.berghei*. Since mortality is greatest 24–48 h after a bloodmeal, it is likely to be due to gut damage caused by penetrating ookinetes, a process which reaches a peak at this time. However, in the presence of antibodies to the midgut components of sugar-fed mosquitoes, the mortality-rate of infected mosquitoes was reduced significantly. It would appear that antibodies to structural components of the midgut protect the mosquito against ookinete penetration. Consistent with this explanation is the observation that, among mosquitoes surviving *P.berghei* infection, a significantly smaller proportion of those fed on mice immunized with sugar-fed midgut components became infected with oocysts compared to those feeding on control mice. It is conceivable that there are specific receptors for ookinetes in the mosquito midgut and antibodies directed against the receptor interfere with the parasite–gut interaction.

Although antibodies to blood-fed midgut tissue appeared to protect mosquitoes against ookinete penetration of the midgut, they were less effective than antibodies against sugar-fed midguts. Midgut antigen preparations of blood-fed mosquitoes contain peritrophic membranes as well as trypsin and other digestive enzymes, which are either absent or present in greatly reduced amounts in sugar-fed midgut preparations. It seems likely that anti-trypsin antibodies (Gass & Yeates, 1979) and anti-peritrophic membrance antibodies may negate an anti-midgut receptor antibody effect in permitting greater numbers of ookinetes to reach the midgut epithelium. Immunization against the head/thorax did not give consistent results on the infectivity of *P.berghei,* although a tendency towards protection against infection was seen. This may be due to the known presence of midgut cross-reacting antibodies in mice immunized with head/thorax (Ramasamy *et al.,* 1988a). However, mice immunized with head/thorax antigen may also have antibodies against mosquito neurosecretory hormones and other components that modulate parasite infectivity. The influences of mosquito hormones on *Plasmodium* infections, if any, have not been documented.

Considerable variation between results obtained with different mosquitoes fed on the same mice (e.g. oocyst numbers in mosquitoes fed on C4, Table 2) and between mosquitoes fed on different mice immunized in the same manner (mortality in D1 and D3 Table 1; C1 and C4a, Table 2) made it more difficult to obtain statistically significant results. Most of the variability is, however, inherent in the complex biology of the experimental system.

Since no differences in either parasitaemias or gametocytaemias were observed between control and immunized mice, it is unlikely that immunization produced a direct effect on the viability of circulating gametocytes. We cannot totally exclude the possibility that the observed transmission blocking effect is due to the weak cross-reaction of some mosquito midgut antigens with the surface antigens of *P.berghei* gametes, zygote or ookinete. However, this would appear to be a less likely explanation in view of the parallel observation that anti-midgut antibodies also block the infectivity of arboviruses to mosquitoes (Ramasamy *et al.,* 1990).

Our observations on the *P.berghei/An.farauti* model suggest that immunization with midgut antigens produces two effects, viz reduction in mosquito infection rates and a reduction in the mortality of infected mosquitoes, that have opposite consequences on the transmission of malaria in the vertebrate population. Unlike the *P.berghei/Anopheles* model (Gad *et al.,* 1979; present observations), however, *P.falciparum* infection of anopheline mosquitoes is not accompanied by high mortality, an observation which may be related to the different routes of midgut penetration (Meis & Ponnudurai, 1987a, b). Therefore it is possible that immunization with mosquito midgut antigens may serve to reduce falciparum malaria transmission in a human population. We suggest that two additional features may improve the effectiveness of mosquito midgut antigens for successful transmission blocking immunity in human malaria. Firstly, the peripheral blood gametocytaemias in man are generally lower than in mice with *P.berghei* and this may make the anti-midgut antibodies more effective in blocking transmission. Also the use of a few relevant target antigens (or portions of them) from the mosquito midgut for immunization may increase the concentration of specific antibodies in the host by several orders of magnitude. This in turn may have a more marked effect on the infectivity of the parasite to the mosquito than was observed in our model. More investigations are clearly needed to evaluate the possibilities for blocking the trans-

mission of human malaria that are opened up by these observations.

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