Jpn. J. Infect. Dis., 55, 78-82, 2002

Original Article

Effect of Anti-Mosquito Hemolymph Antibodies on Fecundity and on the Infectivity of Malarial Parasite *Plasmodium vivax* to *Anopheles stephensi* (Diptera:Insecta)

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(Received March 6, 2002. Accepted July 18, 2002)

SUMMARY: Rabbit antibodies to hemolymph antigens (102.5, 101, 100, 96, 88, 80, 64, 55, 43, 29, and 23 kDa) of *Anopheles stephensi* reduced fecundity as well as viability in *An. stephensi*. However, ingestion of these antibodies was not associated with a marked effect on the engorgement of mosquitoes but egg laying was significantly delayed. Antisera raised against hemolymph proteins were also used to identify cross reactive antigens/epitopes present in other tissues by Western blotting, as well as by in vivo ELISA. In addition, a significant reduction in oocyst development was also observed in *An. stephensi* mosquitoes that ingested anti-hemolymph antibodies along with *Plasmodium vivax*. The results confirmed the feasibility of targeting mosquito antigens as a novel anti-mosquito strategy, as well as confirmed the usefulness of such antigens for the development of a transmission-blocking vaccine.

INTRODUCTION

In spite of numerous studies, anti-arthropod vaccine, an alternative to insecticides, remains in its infancy. Limited success has been achieved to date by vaccination with crude tissue extracts. A range of mosquito antigen preparations has been used for immunizing vertebrate hosts in various vaccine trials, and yet results have rarely been of sufficient efficacy. In addition, putative antigens have not been identified for accurate assessment of the feasibility of this approach (1,2). In our previous reports (2,3), ovary and egg antigens were identified, the antibodies of which reduce the fecundity of *An. stephensi*. Ovarian proteins/insect vitellogens are synthesized by the female fat body (4) and are transferred in the hemolymph; these vitellogens are then selectively sequestered to the developing oocyte by receptor-mediated endocytosis (5) and are stored in yolk granules (6). The present study examines the putative antigenic properties of hemolymph proteins for the first time and demonstrates strong humoral reactivity against hemolymph proteins. Here, we identify the putative antigens and describe the mortality and fecundity of *An. stephensi*. Parasite development was simultaneously blocked in the mosquito that have fed upon them as the next rational step towards obtaining an anti-mosquito and also a transmission-blocking vaccine.

MATERIALS AND METHODS

The culture of the Delhi strain of *An. stephensi* (obtained from Malaria Research Center [MRC], New Delhi, India) was maintained in our laboratory at 28 ± 2°C and 70-80% relative humidity (7). Larvae were reared in bowls at a density of 300 larvae/450 ml of water. Specially prepared cages made of muslin cloth (1 feet by 1 by 1 high) were used for harboring adults, as described previously (2).

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The hemolymph was collected from 40 adult females after 24 h of blood feeding (semi gravid), as described previously (8); collection proceeded by amputation of the antennae and/or by cutting the wings and/or legs to aspirate the hemolymph droplets from the wounds, in phosphate buffered saline (PBS) containing phenylmethylsulfonyl flouride (PMSF). Then, it was centrifuged at 10,000 g for 15 min at 4°C to remove any debris. Trichloroacetic acid (TCA)-precipitated hemolymph proteins (170 μg protein in 0.5 ml 0.1 N NaOH) were injected subcutaneously by emulsification with Freund's complete/incomplete adjuvant in three groups of rabbits, using a previously described method (2).

Immunized rabbits boosted with hemolymph protein antigens from the same group of mosquitoes were used for blood-feeding for periods of up to 6 weeks. Six sets per week (containing about 20 females/set) were generated to observe the egg-laying pattern in *An. stephensi*, as described previously (2). All of the data were subjected to Student's *t* test.

Seventy mosquitoes were dissected to obtain various tissues (hemolymph, midgut, ovary, and salivary gland). The quantity of proteins was estimated by the method of Lowry et al. (9). Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (10) on 10% polyacrylamide gels under reducing conditions. Gels were either silver-stained or were used for overnight electrophoretic transfer to 0.45 μm nitrocellulose membranes at 4°C for the Western blotting (11). Nitrocellulose membranes containing transferred *An. stephensi* hemolymph proteins were blocked with 5% non-fat milk for 1 h. Sheets were incubated with rabbit antisera (1:100) for 1.5 hrs, washed three times with PBS containing 0.1% Tween-20, and then incubated for 1 h at room temperature with Alkaline phosphatase conjugated goat anti-rabbit IgG (1:5000). Bound antibodies were detected by using NBT-BCIP substrate.

Antibody titers in the sera of different groups of rabbits were determined by ELISA using immunizing antigens (10 μg/ml) to coat the wells. Bound antigens were incubated with dilutions of rabbit antisera followed by addition of

Alkaline phosphatase conjugated goat anti rabbit IgG (1:20,000). Immune complexes were detected by a p-nitrophenyl phosphate substrate system.

The cross-reactivity of hemolymph antibodies with other tissues (salivary glands, ovaries, and midgut) from immunized and control female mosquitoes was observed by in vivo ELISA (12). Twenty mosquitoes were dissected to obtain all four of these tissue types after 48 hrs of blood feeding. TCA-precipitated proteins were resuspended in carbonate/bicarbonate buffer, vortexed thoroughly, and were then used to coat ELISA plate wells in triplicate. Bound antigens were incubated with Alkaline phosphatase conjugated goat anti rabbit IgG (1:20,000), as described above.

Sera from immunized rabbits as well as from control rabbits was ingested together with *P. vivax* by *An. stephensi* mosquitoes using a membrane feeding apparatus to screen out the effects of antibodies on parasite development in the mosquitoes. Peak-titer sera from the immunized rabbits was collected, pooled and stored at −70°C until use. Blood samples infected with of *P. vivax* were obtained from patients visiting the MRC. Equal volumes of immune and control sera were mixed with infected blood. Sixty mosquitoes (5-days old) were also separately membrane-fed on immunized and control sera. Unfed or partially fed females were removed. The midgut was dissected in order to count the number of oocysts 8 days after feeding. Similarly, the salivary glands were excised for observation of the presence of sporozoites 14 days after feeding. The percent transmission blockage was determined by the method described by Ponnundurai et al. (13), namely,

$$\frac{\text{Mean oocyst number in controls} - \text{Mean oocyst number in anti-hemolymph}}{\text{Mean oocyst number in controls}} \times 100$$

RESULTS

High antibody titer was detected ranging from $1:10^4$ in the first week to $1:10^6$ in the third week in immunized rabbits, whereas control rabbit serum and pre-immune serum showed a negligible amount of antibodies (below threshold), i.e., titers up to only 10^2. The level of antibody titer decreased during subsequent weeks and showed a minimum value ($1:10^3$) during the sixth week (Fig. 1).

Immunoblotting of anti-hemolymph proteins antibodies recognized 11 different antigens of molecular weight 102.5, 101, 100, 96, 88, 80, 64, 55, 43, 29, and 23 kDa (Fig. 2). Five antigens, 102.5, 88, 64, 55, and 29 kDa were present exclusively in the hemolymph; however, two antigens (43 and 23 kDa) showed cross-reactivity with all of the four tissues. It was also observed that a 101 kDa antigen was also present in the midgut and eggs, and 100 and 96 kDa strands were present in both ovarian tissue as well as in midgut tissue. The 80 kDa strand cross-reacted only with ovarian tissue.

The maximum reduction in fecundity (47.07%) and increase in mortality (33.3%) was observed during the third and fourth weeks, respectively, after the last booster. Thereafter, the rate of reduction in fecundity (13.3%) and the increase in mortality declined continuously until the sixth week, when the last booster was given. (Figs. 3, 4). Total hatchability of mosquito eggs laid by insects fed on immunized sera was also reduced by 47.5% ($P < 0.001$). The corresponding body weight after engorgement decreased by 9.2% during the first week of the study ($P < 0.05$). However, this difference in engorgement was statistically insignificant during the subsequent weeks (Table 1). It is of note that egg laying was also delayed by about 12 hrs in the mosquitoes fed upon immunized blood until the fourth week.

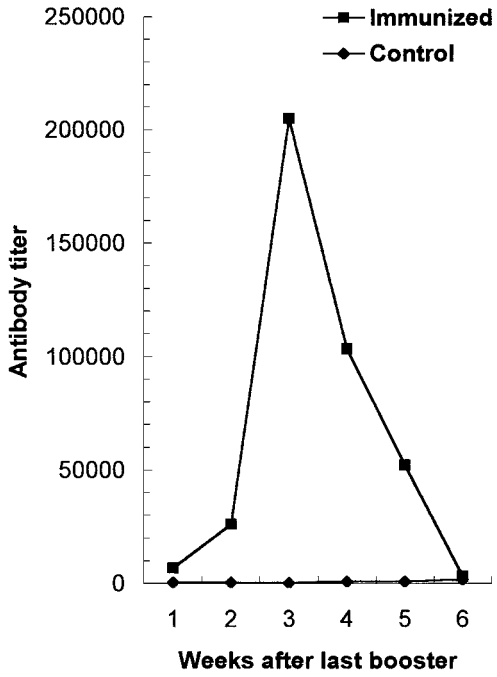

Fig. 1. Antibody titer after last booster measured by antibody capture ELISA.

An ELISA-based assay was used to demonstrate the binding of blood-fed polyclonal antibodies to different tissues (Fig. 5). Much higher binding was detected as compared to the control blood-fed. These results demonstrate that the antibodies fed to the mosquitoes could traverse through the midgut wall and reach their target. The cross-reactivity of these antibodies was observed with other tissues in findings that were also confirmed by Western blotting.

In comparison to the control group, a significant reduction

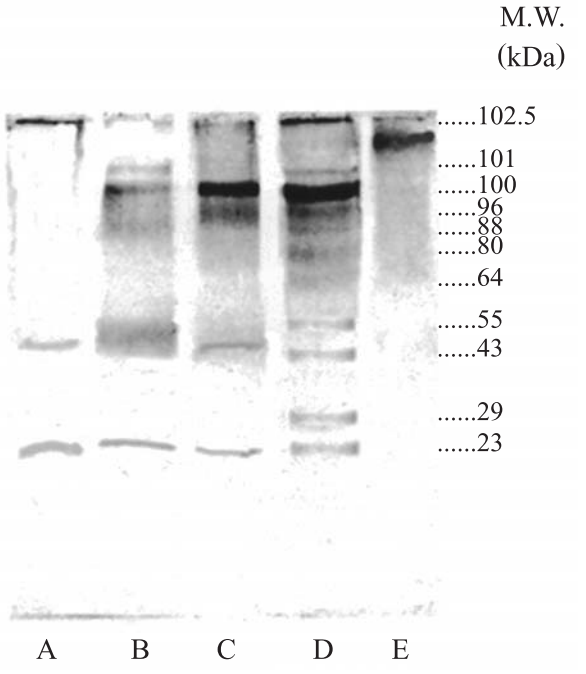

Fig. 2. Western blot analysis of specific tissue expression of antigenic polypeptides.
A: Salivary glands, B: Midgut, C: Ovary, D: Hemolymph, E: Eggs.

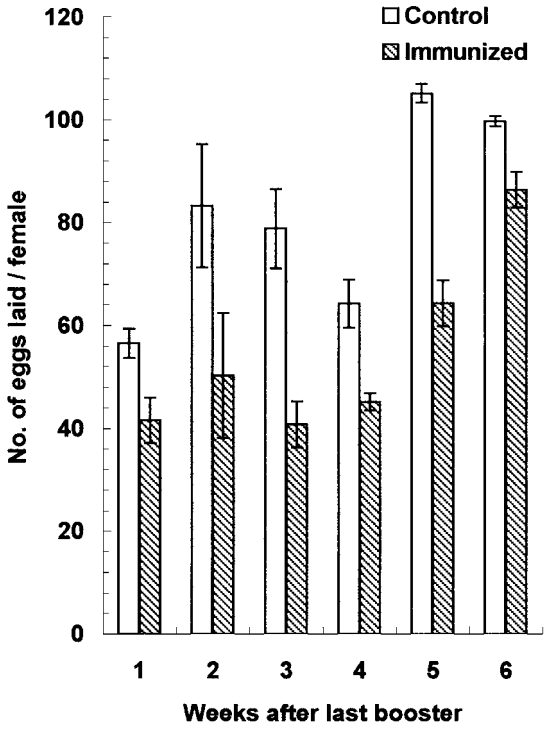

Fig. 3. Effect on fecundity of *An. stephensi* fed on the blood of rabbits immunized with hemolymph antigens of *An. stephensi*.

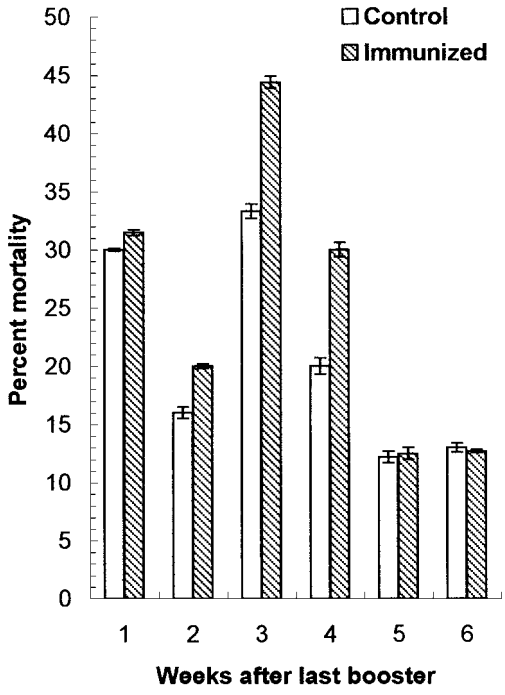

Fig. 4. Effect on mortality of *An. stephensi* fed on the blood of rabbits immunized with hemolymph antigens of *An. stephensi*.

Table 1. Effect of anti-mosquito hemolymph antibodies on fecundity, hatchability, and viability of *An. stephensi*

Weeks after last booster	Reduction in fecundity (%)	Engorgement (mg)		Hatching /female		Reduction in hatchability (%)	Increase in mortality (%)
		C	I	C	I		
1	26.37***	0.98 (±0.059)	0.89** (±0.028)	39.5 (±0.62)	23.16 (±3.5)	41.3***	4.76*
2	39.6**	0.93 (±0.07)	0.85 (±0.016)	66.6 (±10.0)	36.3 (±8.05)	45.4***	20.0***
3	47.07***	0.97 (±1.01)	0.92 (±0.37)	63.0 (±5.9)	31.06 (±7.1)	47.5***	25.0***
4	29.9*	0.92 (±0.51)	0.91 (±1.1)	53.1 (±1.2)	33.6 (±4.1)	36.7**	33.3***
5	38.7***	0.88 (±0.03)	0.90 (±0.08)	88.5 (±6.5)	49.8 (±2.0)	43.7***	2.4
6	13.3	0.82 (±0.04)	0.83 (±0.14)	84.2 (±1.0)	67.4 (±7.7)	19.9*	—

*Significant at $P < 0.1$.
**Significant at $P < 0.05$.
***Significant at $P < 0.001$.

Table 2. Effect of anti-mosquito hemolymph antibodies on malaria parasite development in mosquito *An. stephensi*

No. of mosquitoes /group		Infected mosquitoes (%)		Mean no. of oocysts/mosquito		Transmission blocking (%)
Control	Anti-HL	Control	Anti-HL	Control	Anti-HL	
59	56	73.8	55.8	139.5 ±5.1	32.5 ±3.9	76.7*

*Significant at $P < 0.05$.

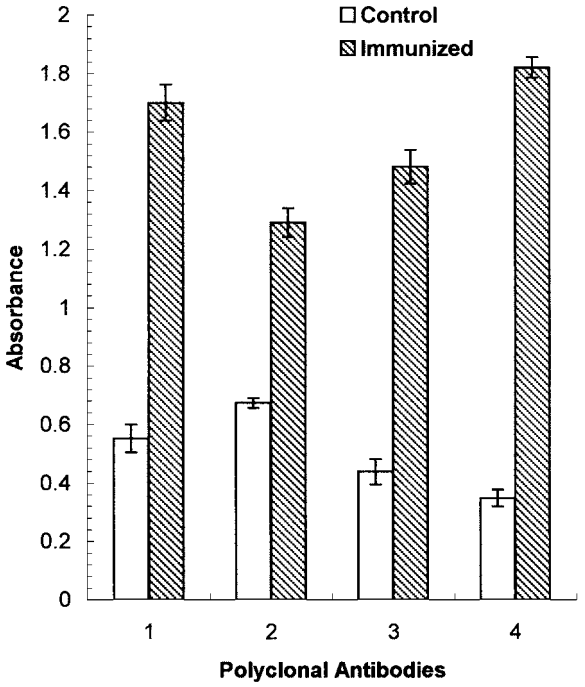

Fig. 5. In vivo ELISA of anti-hemolymph antibodies to various tissues of *An. stephensi*.
1: Salivary glands, 2: Midgut, 3: Ovary, 4: Hemolymph.

in parasitic infection was observed in *An. stephensi* that had ingested anti-hemolymph antibodies along with *P. vivax* (Table 2). The infection rate of *An. stephensi* was reduced by about 18% when mosquitoes had fed on immune sera. Moreover, the mean number of oocysts per infected mosquito was also reduced drastically by about 76.7% ($P < 0.05$). However, the infection rate with sporozoites was also reduced by 23% (data not shown).

DISCUSSION

The efficacy of antibodies raised against the hemolymph proteins of semi-gravid female mosquitoes was considered together with a previous study of the development of antibody responses (2,3). These studies form the foundation for the characterization of anti-mosquito antibodies, especially anti-hemolymph antibodies, which were studied for the first time. The observed reduction in fecundity as well as viability was in accordance with the results of previous studies (14-16). However, prior studies made use of crude antigen preparations, in which either the whole body or the head, thorax, and abdomen were used separately. Almeida and Billingsley (17) have also discussed the effects of induced immunity against *An. stephensi* on its survival and fecundity. However, the reduction in fecundity observed in that study was merely 18%, 16%, 21%, and 22% when mosquitoes fed on antibodies to head, gut, ovaries, and fat bodies, respectively. Recently, Gakhar et al. (2) also showed ≤ 66% reduction in the fecundity of *An. stephensi* when the mosquitoes had fed upon anti-mosquito ovary antibodies. The maximum reduction in fecundity and viability during the third and fourth weeks, respectively, after the last booster, coincides with the results of ELISA. A high ELISA titer ($1:10^5$-10^6) is also in accordance with the results of a study by Almeida and Billingsley (1), who also demonstrated the progression of immune responses in mice during five sequential immunizations with *An. stephensi* mosquito extracts. However, monoclonal and polyclonal antibodies to vitellin alone did not affect the fecundity of *Aedes Aegypti* (16), indicating that other target antigens were also involved.

Fecundity was adversely affected in two ways, namely, by reduction and delay. A delay of 12 hrs was observed in the group for which the blood meals had antibodies against hemolymph proteins. A similar delay in egg laying was also observed by Almeida and Billingsley (17) in the case in which mosquitoes were fed blood with anti-fat body antibodies. The fat bodies act as site for synthesis of vitellogenins, which are secreted into the hemolymph in order to transport them to the developing oocytes in the ovary. Therefore, the antibodies against either of these two may affect egg laying in both ways, i.e., reduction as well as delay. However, the mechanisms causing these events remain open to speculation.

Western blotting of hemolymph antibodies revealed four polypeptides, i.e., 80, 64, 43, and 29 kDa, which were also observed in the case involving anti-mosquito ovary antibodies (2). One polypeptide of 100 kDa was also observed with anti-mosquito egg antibodies (3). However, Brennan et al. (12) have suggested that this 100 kDa polypeptide is a vitellogenin, which appears only after a blood meal in *An. stephensi*.

With this information in mind, we carried out an in vivo binding assay in order to ascertain the role of anti-hemolymph protein antibodies as putative candidates for an anti-mosquito vaccine. We determined by in vivo ELISA that anti-hemolymph antibodies taken by mosquitoes through a blood meal are indeed capable of binding with hemolymph and, to some extent, to other tissues. Cross-reactivity was extensive and was attributed to different causes; antigens or epitopes may be common to other tissues, or non-specific binding may also occur with low-affinity antibodies.

The present study also demonstrated that antibodies against hemolymph antigens, when ingested by mosquitoes along with infected blood, affected the development of oocysts in the midgut and/or the migration of sporozoites to the salivary glands. These results support the findings of Lal et al. (18) and Brennan et al. (12), who also observed the same phenomena.

Vector control has become increasingly difficult due to the development of insecticide resistance. Moreover, only limited funds are available for continuous redistribution for the purpose of addressing the diversifying health needs of regions with high malaria endemicity. Therefore, new strategies of malaria control are currently required. Immune responses against concealed antigens (in internal organs which were never exposed to the host during blood feeding) can reduce the longevity and fecundity of mosquitoes. The anti-mosquito effects achieved to date are of limited success compared to those associated with the tick vaccine (19). This difference can be attributed to differences in the digestive physiology of these organisms, as well as to the length of time spent at the host for blood feeding. A mosquito vaccine approach could be extremely effective, provided that the vector number were reduced, and parasitic transmission were blocked either directly (18,20) or else via reducing vector longevity (21). Although the results of the present study are preliminary, they do demonstrate that *An. stephensi* hemolymph proteins can act as an additional candidate for/putative means of anti-mosquito activity, as well as acting as a transmission blocking vaccine.

ACKNOWLEDGMENTS

The authors are grateful to the University Grants Commis-

sion for the financial assistance received for this project. In addition, Dr. T. Adak, Deputy Director, Malaria Research Centre, is to be thanked for providing the mosquito culture.

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