Induced Immunity against Haemolymph Proteins of Anopheles stephensi: Effects on Fecundity and Transmission Blocking of Malaria Parasite

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Rabbit antibodies to nine antigens (100, 88, 80, 64, 55, 43, 29, 23 and 15 kDa), derived from haemolymph of Anopheles stephensi mosquitoes after first gonotrophic cycle, tended to reduce the number of eggs produced by the mosquitoes and block the transmission of malaria parasite, Plasmodium vivax. Cross-reactivity of these antibodies was checked both by Western blotting and in vivo ELISA. In addition, effect of these antibodies on mortality, engorgement and hatchability in mosquitoes was also determined. The results indicated that haemolymph antibodies have the potential to disrupt the reproductive physiology of mosquitoes and further studies are needed with the target antigens.

Keywords: Anopheles, haemolymph, antibodies, parasite, transmission-blocking

Introduction
The recent surge in malaria cases, due to spread of multi-drug resistant parasites and emergence of insecticide-resistant anopheline mosquitoes, has emphasized the need for alternative malaria control strategies. Consequently, the research efforts towards the development of vaccines got a boost. Of the several attempts to develop vaccines against blood feeding arthropods so far, only anti-tick vaccine (Bm 86) has been successful at commercial level (Willadsen et al, 1989). Also, effects of anti-mosquito antibodies have been variable and seldom sufficient to have appreciable biological effect (Kay & Kemp, 1994; Jacobs-Lorena & Lemos, 1995; Willadsen & Billingsley, 1996). In our previous reports, however, ovarian and egg antigens of Anopheles stephensi have been identified, and their antibodies were able to reduce the fecundity and viability of eggs. They also inhibited the development of Plasmodium vivax in mosquitoes (Gakhar et al, 2001; Gakhar & Gulia, 2001).

Ovarian proteins/insect vitellogenins are synthesized by the female fat body (Dhadialla & Raikhel, 1990) and secreted in the haemolymph, which are then selectively sequestered to the developing oocyte by receptor mediated endocytosis (Giorgi et al, 1998) and stored in yolk granules (Raikhel & Dhadialla, 1992). Therefore, the aim of present study was to identify protective antigens present in the haemolymph of female A. stephensi, particularly after egg laying to optimize the conditions for their wider use.

Materials and Methods

Maintenance of Culture
The culture of Delhi strain of A. stephensi mosquitoes (obtained from Malaria Research Centre, New Delhi) was maintained in the laboratory at 28±2°C and 70-80% RH (Gakhar et al, 1997). Larvae were reared in bowls at a density of 300 larvae/450 ml of water. Specially prepared cages made up of muslin cloth (0.3×0.3×0.3 m³) were used for harbouring adults as described earlier (Gakhar et al, 2001).

Immunization Schedule
The haemolymph was collected from 7-day-old 40 adult female mosquitoes after egg laying as described earlier (Gakhar & Shandilya, 2000) in phosphate buffered saline (PBS) containing PMSF. It was then centrifuged at 10,000 X g for 15 min at 4°C to remove any debris. TCA precipitated haemolymph proteins (0.5 ml, 160 µg protein) were injected subcutaneously by emulsifying with Freund's complete/incomplete adjuvant in a group of three rabbits in the manner described earlier (Gakhar et al, 2001). In parallel, control rabbit was immunized with PBS+Freund's complete/incomplete adjuvant in the same manner.
**Survival and Fecundity Assay**

Control and immunized rabbits, boosted with haemolymph protein antigens, were used for blood feeding by mosquitoes up to 8 weeks on alternate days. Six sets per week, containing about 20 female mosquitoes/set, 4-day-old, were used to observe the egg-laying pattern on wet filter papers. Ovaries of females were also examined for the presence of any retained eggs. Total number of eggs was counted by summing up the oviposited and unlaid eggs. The mean number of eggs laid, and per cent reduction in fecundity, hatchability, engorgement and viability were calculated. The data were subjected to students' 't' test.

**Western Blot Analysis**

Seventy mosquitoes were dissected for different tissues, viz. haemolymph, midgut, ovary and salivary glands. The quantity of proteins in them was estimated as per the method of Lowry et al (1951). However, the proteins were separated by SDS-PAGE using 10% polyacrylamide gel under reducing conditions essentially by Laemmli (1970) and as described in detail by Gakhar & Shandilya (1999). Gels were either silver stained or transferred overnight electrophoretically to 0.45 μm nitrocellulose membrane at 4°C for western blotting (Towbin et al., 1979). Nitrocellulose membrane containing transferred haemolymph proteins were blocked with 5% non-fat milk. Sheets were incubated with rabbit antisera (1:100) for 1½ hrs, washed with PBS containing 0.1% Tween-20 and then incubated for 1 hr at room temperature with Horse radish peroxidase conjugated goat anti-rabbit IgG (1:5000). Bound antibodies were detected by using DAB-H_2O_2 substrate.

**Antibody Titre Analysis**

Antibody titre in serum of different groups of rabbits was determined by ELISA using immunizing antigens (10 μg/ml) to coat the wells. Bound antigens were incubated with dilutions of rabbit antisera followed by alkaline phosphatase conjugated goat anti-rabbit IgG (1:20,000). Immune complex was detected by p-nitrophenyl phosphate substrate system. ELISA results were extrapolated from endpoint titre, which was defined as the highest dilution of the serum that yielded an absorbance value above that achieved with the same dilution of control serum.

The in vivo binding and cross-reactivity of haemolymph antibodies with other tissues (haemolymph, salivary glands, ovary and midgut) from immunized and control female mosquitoes was observed by ELISA (Brennan et al., 2000). Twenty mosquitoes were dissected out for all the four tissues after 48 hrs of blood feeding. TCA precipitated proteins were resuspended in carbonate/bicarbonate buffer, vortexed thoroughly, and 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.

**Rocket Immunoelectrophoresis**

Different developmental stages, viz. larvae, pupae, adult males, adult females and haemolymph from female mosquitoes after egg laying, were used to coat the wells for rocket immunoelectrophoresis in 1% agarose gel having anti-mosquito haemolymph antibodies (Laurell, 1972).

**Parasite Invasion Blocking Assay**

Sera from immunized rabbits as well as from control were ingested along with P. vivax to mosquitoes by membrane feeding to screen out the effect of antibodies on parasite development in mosquitoes. Peak-titre sera from rabbits were collected, pooled and stored at -70°C for the use in membrane feeding. The gametocytes of P. vivax were obtained from patients. Parasitaemia and gametocytaemia were determined by examining Giemsa-stained blood smears. Blood samples of patients diluted in 10 volumes of suspended animation solution (10 mM Tris, 170 mM NaCl, 10 mM glucose, pH 7.4) were centrifuged at 500 X g for 10 min at room temperature. The plasma was removed and parasitized human erythrocytes were mixed with equal volume of immune and control sera in the manner described by Srikrishnaraj et al (1995) for the membrane feeding of mosquitoes. Sixty mosquitoes (5-day-old) were membrane fed separately on immunized and control sera. Unfed or partially fed females were removed. After 8 days, midgut was dissected out for counting the number of oocysts. Similarly, after 14 days, salivary glands were dissected out for observing the presence of sporozoites. Per cent transmission blocking was determined by the following method of Ponnundurai et al (1987):

\[
\text{Mean oocysts in control} - \text{Mean oocysts in anti-mosquito haemolymph} \times 100 \\
\text{Mean oocysts in control}
\]
Results

High antibody titre, ranging from 1:10^4 in first week to 1:10^6 in 3rd week, was detected in immunized rabbits whereas control rabbit serum and pre-immune serum showed negligible amount of antibodies (below threshold), i.e. titre up to 10^2 only. The level of antibody titre decreased during subsequent weeks and showed the least (1:10^3) during 8th week (Fig. 1).

Serum from control rabbits gave no precipitin line with any antigen in double diffusion test. However, haemolymph antigens gave a prominent precipitin line with antiserum. Immunoblotting of haemolymph proteins recognized 9 antigens of molecular weight 100, 88, 80, 64, 55, 43, 29, 23 and 15 kDa (Fig. 2). Of these, three antigens, 80, 23 and 15 kDa were present exclusively in haemolymph; whereas, antigen, 29 kDa was present in all the four tissues. Similarly, antigens, 64, 55 and 43 kDa were present in haemolymph and ovary; 88 kDa in ovary and salivary gland; and 100 kDa in both midgut and salivary gland.

The Rocket immunoelectrophoresis shows 0.5 mg/ml of haemolymph and 0.18 mg/ml of adult female antigens binding with the antiserum raised against haemolymph proteins. However, in other stages no rocket was formed (Fig. 3). The Rocket immunoelectrophoresis with control and pre-immune serum also did not show any rocket formation. In case of developmental stages and adult male, absence of rocket formation shows that the specific antigens were either absent or having very little cross-reactivity. However, a prominent peak was observed in case of adult female showing the female specificity of these antigens.
Table I—Effect of anti-mosquito haemolymph (after egg laying) antibodies on fecundity, hatchability and viability of A. stephensi.

<table>
<thead>
<tr>
<th>Weeks after last booster</th>
<th>Reduction in fecundity (%)</th>
<th>Engorgement (mg)</th>
<th>Hatching/female</th>
<th>Reduction in hatchability (%)</th>
<th>Mortality/female</th>
<th>Increase in mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>I</td>
<td>C</td>
<td>I</td>
<td>C</td>
<td>I</td>
</tr>
<tr>
<td>1</td>
<td>39.26***</td>
<td>0.96 (±0.06)</td>
<td>0.85** (±0.03)</td>
<td>44 (±8.3)</td>
<td>31.7 (±7.5)</td>
<td>28*** (±0.8)</td>
</tr>
<tr>
<td>2</td>
<td>38.3***</td>
<td>1.0 (±0.08)</td>
<td>0.92* (±0.02)</td>
<td>49.6 (±2.9)</td>
<td>28.8 (±1.1)</td>
<td>42*** (±0.81)</td>
</tr>
<tr>
<td>3</td>
<td>44.4***</td>
<td>0.97 (±1.0)</td>
<td>0.92 (±0.4)</td>
<td>50.7 (±4.7)</td>
<td>28.3 (±4.8)</td>
<td>44.2*** (±1.1)</td>
</tr>
<tr>
<td>4</td>
<td>39.0***</td>
<td>0.92 (±0.5)</td>
<td>0.91 (±1.1)</td>
<td>48.2 (±2.1)</td>
<td>30.3 (±2.5)</td>
<td>37.2** (±0.6)</td>
</tr>
<tr>
<td>5</td>
<td>21.4***</td>
<td>0.88 (±0.09)</td>
<td>0.90 (±0.08)</td>
<td>47.7 (±5.2)</td>
<td>38.2 (±3.5)</td>
<td>19.9** (±1.02)</td>
</tr>
<tr>
<td>6</td>
<td>19.4**</td>
<td>0.83 (±0.14)</td>
<td>0.82 (±0.04)</td>
<td>46.1 (±1.3)</td>
<td>37.2 (±0.34)</td>
<td>19.0*** (±2.5)</td>
</tr>
<tr>
<td>7</td>
<td>19.3**</td>
<td>0.92 (±0.03)</td>
<td>0.91 (±0.08)</td>
<td>50.8 (±1.2)</td>
<td>42.9 (±1.7)</td>
<td>15.5** (±0.8)</td>
</tr>
<tr>
<td>8</td>
<td>11.9</td>
<td>0.92 (±0.4)</td>
<td>0.92 (±1.2)</td>
<td>45.3 (±4.8)</td>
<td>42.8 (±6.9)</td>
<td>5.5</td>
</tr>
</tbody>
</table>

***Significant at p<0.001  C- Control, I- Immunized  **Significant at p<0.01  *Significant at p<0.1

The maximum reduction in fecundity (44.4%) and hatchability (44.2%) of the eggs laid by the mosquitoes fed on immunized sera was observed during 3rd week after the last booster (p<0.001) (Table I & Fig. 4). Thereafter, the rate of reduction declined continuously up to 8th week of the last booster (fecundity, 11.9%; hatchability, 5.5%). Similarly, an increase of only about 16-17% in the mortality of females was observed up to 2nd week after the last booster, which sharply declined in the 3rd week and thereafter no differences were observed between females fed on immunized sera and control. The maximum reduction in corresponding body weight after engorgement was about 8% during 2nd week (p<0.1). However, this difference became statistically insignificant during the subsequent weeks. It was interesting to note that the egg laying was also delayed by about 12 hrs in the mosquitoes fed upon immunized blood up to 3rd week and by about 8 hrs during 4-6th week, thereafter no delay was recorded (Personal observation).

An ELISA based assay was used to demonstrate the binding of polyclonal antibodies ingested along with the blood to different tissues (Fig. 5). The results demonstrated that fed antibodies traversed through the midgut wall and reached their target. However, the...
cross-reactivity of these antibodies was also observed with other tissues by Western blotting.

Significant reduction in the parasite infection was observed in females that ingested anti-mosquito haemolymph antibodies along with P. vivax as compared to control group (Table 2). The infection rate of females was reduced by c. 19% in case of those fed on immune sera. Moreover, the mean number of oocysts per infected mosquito was also reduced drastically by c. 71.6% (p<0.05); whereas, the number of sporozoites per infected mosquito rate was reduced by c. 21% (p<0.05).

Discussion

Anti-mosquito antibodies against 'concealed' antigens have long been postulated to be able to reduce the fecundity of mosquitoes (Ramasamy et al, 1988). In the present study, reduction in fecundity and viability of eggs in A. stephensi mosquitoes fed on haemolymph antibodies is in accordance with the previous studies (Alger & Cabrera, 1972; Sutherland & Ewen, 1974; Ramasamy et al, 1988). However, they used crude preparation of antigens, either using the whole body, or head, thorax and abdomen separately. Almeida and Billingsley (1998) have also reported the effect of induced immunity on survival and fecundity of mosquitoes but the reduction in fecundity observed by them ranged between 16-22% only. Recently Gakhar et al (2001) also showed up to 57.7 and 50.9% reduction in fecundity in A. stephensi when fed upon anti-mosquito ovary and egg antibodies, respectively (Gakhar & Gulia, 2001). In the present study, the maximum reduction in fecundity and viability during 3rd week after last booster also coincides with high ELISA titre (10^6). Almeida and Billingsley (1999) have also shown the progression of immune responses in mice during five sequential immunizations with extracts.

Egg laying in A. stephensi mosquitoes was adversely affected in two ways, i.e. by reduction and delay. A delay of 12 hrs was observed in case of blood meal having antibodies against haemolymph proteins. A similar delay in egg laying was also observed by Almeida and Billingsley (1999) in case of mosquitoes fed upon blood having anti-fat body antibodies. The fat bodies act as a site for synthesis of vitellins, which are secreted into haemolymph to reach up to developing oocytes in the ovary. Therefore, the antibodies against either of these two may affect the egg laying in both ways, i.e. by reduction and delay. The reduction in number of eggs produced by A. stephensi may be attributed to specific anti-mosquito antibodies binding to target antigens and disrupting the normal physiology of mosquitoes. The mechanism of anti-mosquito response could be one or a combination of several factors, i.e. fat body synthesis may be down regulated, the uptake of circulatory vitellogenins may be inhibited or the content of some of developing follicles may be reabsorbed. Alternatively, the presence of antibodies in ingested blood might act quickly to irritate the gut

### Table 2—Effect of anti-mosquito haemolymph (after egg laying) antibodies on malarial parasite development in A. stephensi

<table>
<thead>
<tr>
<th>No. of mosquitoes/group</th>
<th>Infected mosquitoes (%)</th>
<th>Mean no. of oocysts/mosquito</th>
<th>Transmission blocking (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Anti-HL</td>
<td>Control Anti-HL</td>
<td>Control Anti-HL</td>
<td>Control Anti-HL</td>
</tr>
<tr>
<td>53</td>
<td>47</td>
<td>68.3</td>
<td>49.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(±4.2)</td>
<td>(±3.1)</td>
</tr>
</tbody>
</table>

*Significant at p<0.05 Anti-HL: Anti-mosquito haemolymph antibodies
thereby reducing the total intake of blood. However, the exact mechanism is open to speculation.

Western blotting of haemolymph antibodies revealed four polypeptides, viz., 80, 64, 43 and 29 kDa. These polypeptides were also observed in case of anti-mosquito ovary antibodies (Gakhar et al, 2001) and one 100 kDa was also observed in anti-mosquito egg antibodies (Gakhar & Gulia, 2001). Presence of these polypeptides in both haemolymph and ovary suggests that they may be having a direct role in oocyte development.

In vivo ELISA study demonstrated that anti-mosquito haemolymph antibodies fed to mosquitoes through blood meal are indeed capable of binding with haemolymph and also up to some extent to other tissues. Cross-reactivity was extensive and may be attributed to different causes; antigens or epitopes may be common to other tissues, or non-specific binding by low affinity antibodies may also occur.

The present study also indicated that antibodies against haemolymph antigens, when ingested by mosquito along with infected blood, affect the development of oocytes in the midgut and/or translocation of sporozoites into the salivary glands. These results are in conformity with Lal et al (1994) and Brennan et al (2000); they also observed the same phenomenon with anti-mosquito antibodies.

It is clear from the present study that high-titre antibodies against a specific combination of antigens may be more effective in bringing about a disruption of mosquito physiology. However, this possibility requires further investigation using combination of monospecific polyclonal and monoclonal antibodies produced against purified antigens. Although, these results are of preliminary nature they open new avenues for the development of anti-mosquito and transmission-blocking vaccines.

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References