Midgut antibodies reduce the reproductive capacity of *Anopheles stephensi* (Diptera: Culicidae)

S K Gakhar* & Amita Suneja

Department of Biosciences, Maharshi Dayanand University, Rohtak 124 001, India

and

T Adak

Malaria Research Centre, New Delhi, 110 054.

Received 18 June 2004; revised 12 January 2005

Rabbits immunized with polypeptides of midgut of glucose fed *A. stephensi* resulted in high titer of antibodies \((10^4 - 10^6)\) as detected by ELISA. Effect of antisera on fecundity, hatchability and engorgement was investigated. Fecundity was reduced drastically (62.4%). Eight polypeptides were recognized by the antisera raised against midgut tissues viz., 92, 85, 55, 52, 45, 38, 29 and 13 kDa. Cross reactivity of these antibodies with different tissues of *A. stephensi* as well as different species of *Anopheles* was also analyzed. The results indicated that anti-mosquito midgut antibodies had the potential to disrupt the reproductive physiology of mosquitoes in view of the present study, there is a need for further investigation with target antigens.

**Keywords:** *Anopheles stephensi*, Anti-mosquito, Malaria, Midgut, Vaccine

Epidemic of malaria caused by drug resistant parasite and insecticide resistant vector has lent particular urgency to the development of malaria vaccine. Immunological control as an alternative to insecticides has been successful in development of anti-tick vaccine. Despite various attempts made, a comparable anti-mosquito vaccine development remains elusive. However, recent results are encouraging.

The present investigation has been carried out on *Anopheles stephensi*, an important vector of malaria parasite in Indian sub-continent where its control has been hampered by insecticidal resistance. Mosquito midgut was the obvious tissue as a source of immunizing antigens for vector vaccine targeting, because it has direct contact both with antibodies present in the blood meal and with parasite.

Effect of antibodies developed in immunized rabbit was studied on the fecundity, mortality and engorgement of mosquitoes. In addition, we have also identified putative antigens which block the parasite development in the mosquitoes. The cross reactivity of the serum has also been tested with different tissues of *A. stephensi* as well as with different *Anopheles* species.

*Correspondent author:
E-mail: gakharsk@yahoo.co.in

**Materials and Methods**

The culture of *A. stephensi* (Delhi strain, Malaria Research Centre, New Delhi) was maintained in laboratory at 28±2°C and 70-80% relative humidity (RH) as described earlier. Midguts from glucose fed female mosquitoes (about 70; 4 days old) were dissected out in ice-cold phosphate buffer saline (PBS) containing 0.2 mM of phenyl methyl sulfonyl fluoride (PMSF). The tissues were centrifuged at 10,000 x g for 15 min at 4°C after homogenization. The supernatant was used as an immunizing antigen. The protein concentration was determined by the method as described by Lowry et al.

Polyclonal antibodies were produced by immunizing white rabbits (New Zealand) with midgut homogenate (0.5 ml containing 0.8 mg protein.) after emulsifying it with equal volume of Freund’s complete/incomplete adjuvant sub-cutaneously at multiple sites in a group of three rabbits as described earlier. In parallel, control rabbit was immunized with PBS + Freund’s complete/incomplete adjuvant in same manner.

Antibody titer was measured by serial dilution of antisera by PBS *in vitro* ELISA using midgut proteins (10 µg/well) to coat the wells of ELISA plate. Proteins were obtained from 4 days old mosquito gut. The plate was incubated with gut proteins at 37°C for
1 hr and then at 4°C overnight. This was followed by blocking for 1 hr at room temperature with 5% non-fat dry milk. Bound antigens were incubated with appropriate dilutions of rabbit serum for about 1½ hr and the wells were washed with PBS-Tween-20, followed by incubation with alkaline phosphatase conjugated goat anti-rabbit IgG (1:20,000) for about 1½ hr at room temperature. The immune complex was detected with p-nitrophenol phosphatase substrate. All ELISA results were extrapolated from endpoint titer, 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.

Soluble proteins were separated by SDS-PAGE using thick slab gels (Imm) containing 10% of acrylamide and a tris-glycine (pH 8.6) buffer system. Tissues were loaded in duplicate. One half of the gel was silver stained and other half was transferred electrophoretically to nitro-cellulose membrane (0.45 µm thick) for Western blotting. Nitrocellulose membrane containing transferred proteins of different tissues and different species of mosquito were blocked with 5% of non-fat milk. Sheets were incubated with anti-midgut antisera raised (1:100) for 1 hr, washed with PBS containing 0.1% Tween-20 and then incubated for 1 hr with alkaline phoshatase conjugate goat anti-rabbit IgG (1:5000). Bound antibodies were detected by using NBT/BCIP substrate.

Immunized rabbits boosted with midgut antigens were used for blood feeding up to 8 weeks. Three sets (weekly), containing about 20 mosquitoes, were made to observe the egg-laying pattern in *A. stephensi* as described earlier. The mean no. of egg laid, percentage reduction in fecundity, hatchability, engorgement and viability were calculated. The data was subjected to Student's t test.

**Results**

High titers of antibodies (ranged from $10^4$-$10^6$) were detected in the rabbit immunized with the midgut antigens, whereas control rabbits immunized with Freund’s adjuvant and PBS showed negligible amount of antibodies (titer up to $10^2$), probably because of stimulation of cross reactive rabbits antibodies by the adjuvant. Antibody titer increased up to third week after last booster and then started declining in subsequent weeks in case of immunized rabbit (Fig. 1). The high antibodies level interferes in the mosquito biology as evidenced on challenge when significant reduction in fecundity was recorded.

Immunoblotting of the midgut proteins of glucose fed mosquitoes recognized with the maximum titer sera eight polypeptides viz., 92, 85, 55, 52, 45, 38, 29 and 13 kDa (Fig. 2). The sera also showed cross reactivity with the proteins of the other tissues of *A. stephensi*. Two polypeptides 29 and 13 were common to haemolymph, salivary gland and ovary. However, 92 and 85 kDa were present in haemolymph, 45 kDa in haemolymph and ovary, and 55 kDa in ovary, whereas 52 and 38 kDa were...
exclusively present in the midgut. Antiserum raised against the midgut of glucose fed mosquitoes was also used to identify the immunogens present in other Anopheles species (Table 1).

There was no statistically significant difference in engorgement and mortality. However, reduction in fecundity was 26.75% (P<0.001) in first week after last booster, which increased up to 62.4% (P<0.01) in third week and subsequently started decreasing and dropped to 4.4% in eighth week (Fig. 3). Reduction in hatchability was 24.5% in first week, 43.5% in third week and dropped to 8% in eighth week (Table 2).

**Discussion**

Immunization trials conducted in the present study using homogenate of midgut tissue were based on the concept that mosquito feeding on appropriately immunized host ingests antibodies specific for target antigens within the mosquito (concealed antigens), producing deleterious effects on the feeding, reproductive performances of mosquitoes and on

<table>
<thead>
<tr>
<th>Weeks after last booster</th>
<th>Reduction in fecundity (%)</th>
<th>C</th>
<th>I</th>
<th>C</th>
<th>I</th>
<th>C</th>
<th>I</th>
<th>C</th>
<th>I</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>26.75***</td>
<td>1.2(±0.2)</td>
<td>1.16(±0.2)</td>
<td>47.45 (±3.3)</td>
<td>35.8 (±4.5)</td>
<td>24.5** (±1.0)</td>
<td>5</td>
<td>7</td>
<td>28.5</td>
</tr>
<tr>
<td>2</td>
<td>38.0**</td>
<td>1.00</td>
<td>1.00</td>
<td>52.5</td>
<td>34.7</td>
<td>34** (±1.8)</td>
<td>10</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>62.4***</td>
<td>1.01 (±0.1)</td>
<td>1.02 (±0.1)</td>
<td>65.0</td>
<td>36.7</td>
<td>43.5*** (±0.8)</td>
<td>10.3</td>
<td>13.3</td>
<td>23.1</td>
</tr>
<tr>
<td>4</td>
<td>40.9***</td>
<td>1.01 (±0.0)</td>
<td>1.01 (±0.0)</td>
<td>68.0</td>
<td>42.8</td>
<td>37*** (±2.9)</td>
<td>13</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>30.8***</td>
<td>0.95 (±1.0)</td>
<td>0.94 (±1.0)</td>
<td>79.6</td>
<td>63.2</td>
<td>20.6*** (±1.6)</td>
<td>9.1</td>
<td>10.1</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>24.0***</td>
<td>1.03 (±1.0)</td>
<td>1.04 (±1.0)</td>
<td>55.9</td>
<td>32.3</td>
<td>14 *** (±1.2)</td>
<td>12.0</td>
<td>13</td>
<td>7.7</td>
</tr>
<tr>
<td>7</td>
<td>10.7***</td>
<td>1.03 (±0.0)</td>
<td>1.03 (±0.0)</td>
<td>62.6</td>
<td>54.0</td>
<td>13.7** (±1.2)</td>
<td>11.0</td>
<td>11.5</td>
<td>4.5</td>
</tr>
<tr>
<td>8</td>
<td>4.4</td>
<td>1.01 (±0.0)</td>
<td>1.00 (±0.0)</td>
<td>62.2</td>
<td>57.2</td>
<td>8* (±1.0)</td>
<td>8</td>
<td>7</td>
<td></td>
</tr>
</tbody>
</table>

Significant at P*** <.0001; ** <.001; * <.05. C-control; and I-immunized.
parasite development. Significant reduction in fecundity of *A. stephensi* was observed that fed on immunized rabbit as compared to that fed on control rabbit. Females from each feeding set were dissected after egg laying and all seemed to have laid their full complement of eggs. The lower fecundity does not seem to be related to reduction in oviposition. This infers that humoral antibodies somehow interfere with the normal process of oogenesis. IgG antibodies have been detected in the haemolymph of anophelines up to 48 hr after blood feeding. Anti-mosquito antibodies against antigen such as midgut tissues, proteases and vitellin have been postulated to be able to reduce the fecundity of mosquitoes. However, monoclonal and polyclonal antibodies to vitellin alone did not affect fecundity of *Ae.aegypti* indicating that other target antigens are also involved.

The mechanism of action of antibodies may be the result of one or combination of several factors like inhibition in metabolism, transport of vitellogenic proteins in the haemolymph, in their uptake by the developing oocytes and upset in hormonal balance, thus affecting may be vitellogenic protein metabolism. High titered antibodies against a specific combination of antigens may be more effective in bringing about a disruption of mosquito reproductive physiology. This possibility requires further investigation using a combination of monospecific polyclonal and monoclonal antibodies produced against purified or synthetic antigens.

The feeding that takes place after immunization can also affect the fecundity and survival of the mosquito. Feeding on rabbits during first week did not affect mosquitoes. However, blood meal of same rabbit caused maximum reduction in fecundity during third week, which incidentally coincided with the maximum titer of antibodies. However, in other studies no such correlation was found between antiarthropod effect and antibody titer.

Earlier studies have also showed marked reduction in fecundity. Ramasamy has reported reduction in fecundity by about 15, 23, and 20% when mosquitoes were fed on antiserum raised against head, thorax and abdomen, respectively. Fecundity of *Ae.aegypti* ingesting anti-head/thorax antibodies was reduced by 35-39% in 2 of the 3 experiments and that of *A.tessellatus* by 15-19%. Recently, 57.7 and 50.9% reduction in fecundity has been reported in *A.stephensi* by anti-ovary and anti-mosquito eggs antibodies, respectively.

We did not observe increased mortality amongst female mosquitoes that had fed on immunized rabbit that was in contrast to the findings of Alger and Cabrera. Anti-midgut antibodies did not affect the longevity also in the present study, which was in accordance with other workers. Differences in the results with some others could be attributed to the amount of the proteins ingested, antibody titers, antigen being derived from blood or glucose fed mosquitoes and immunological tolerance of the mice and rabbit.

Eight antigens were identified by Western blotting. Cross-reactivity with other tissues was also extensive. It can be attributed to epitopes, which may be common in different tissues, or to non-specific binding by low affinity antibodies. Peptides that are recognized will be injected separately in different rabbits to access the efficacy of these peptides individually. Investigations are in progress to determine the role of these polypeptides in artificially induced immunity.

In conclusion, this study showed that rodent antibodies against mosquito midgut antigens when ingested by the mosquitoes caused reduction in fecundity. Clearly, additional work is needed to identify different midgut antigens involved in interacting with parasite individually and their role in reduction of fecundity. Once these proteins have been identified, midgut antigens based anti-mosquito vaccine can be developed for use independently or as a part of multivalent immune intervention strategy.

**Acknowledgement**

The authors are grateful to technical staff, Malaria Research Centre, New Delhi for providing mosquito culture. CSIR is also acknowledged for its financial assistance to the author (AS).

**References**


