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Effects of Anti-Mosquito Salivary Glands and Deglycosylated Midgut Antibodies of *Anopheles stephensi* on Fecundity and Longevity

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Abstract

With the aim of controlling malaria by reducing vector population, the effects of antibodies produced against salivary glands and deglycosylated midgut antigens of *Anopheles stephensi* mosquitoes on fecundity and longevity of the same species were tested. Three deglycosylated preparations of midgut and two preparations of salivary glands were produced, conjugated with aluminum hydroxide gel, and subcutaneously injected to shoulders of TO (Turner Out-bred) mice. After 4 immunizations and assurance of enough antibody production against utilized antigenic suspensions, effects of blood feeding on immunized and control mice were assayed. Insoluble preparation of midgut showed the strongest effect with 23.5% reduction in egg laying, and increasing death rate of vectors in third day after feeding. No significant reduction in fecundity or survivorship was seen with other preparations. *Anopheles* midgut insoluble antigens are potential candidates for designing vaccines against malaria vectors and further investigations need to be done to find effective antigens and the best way of their use.

Keywords: Anopheles stephensi, *Malaria, Longevity, Fecundity, Vaccine*

Introduction

Malaria is a major health problem and annually is responsible for death of more than one million patients (1). Of the most important interventions potentially capable to control malaria, are the vector control programs mostly by using insecticides that got various side effects on the nature and living organisms (2). Since 1972 there has been an increasing interest to employ *Anopheles* antigens to immunize vertebrate hosts in order to decreasing longevity and fecundity of mosquitoes following blood feeding upon such immunized hosts. This kind of Transmission Blocking Vaccines has also given promises of arresting malaria life cycle inside *Anopheles* vectors (3-9).

Anopheles midgut is the first important organ of mosquito that comes in long lasting contact with blood meal bolus and malaria parasites. Like mammalian hosts, in mosquitoes protein molecules lining midgut surface, are masked with oligosaccharides to protect them from proteases (10). These molecules are also receptors for ookinete to be able to pass through midgut cells and form oocyst at the outer surface of the midgut (11). Although it is fascinating to have these molecules as antigens in anti-mosquito or transmission blocking vac-

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cines, as they can be found in mammalian hosts too, it would not be easy to immunize such hosts against them and if possible, autoimmune reactions may harm the host. Deglycosylating midgut glycoproteins, allows immune system to face and produce antibodies directly against bared midgut proteins.

Here we describe the effect of polyclonal antibodies produced against antigenic preparations from midgut and salivary glands of uninfected *An. stephensi* on the same vectors longevity and fecundity. Antigenic preparations of midgut were deglycosylated before being mixed with aluminum hydroxide, the adjuvant, and being injected to mice from TO strain.

Materials and Methods

Mosquitoes *An. stephensi* mosquitoes were reared in the insectaries of the School of Public Health (SPH), Tehran University of Medical Sciences, Iran and Zoology Department of Aberdeen University, Scotland. Adults were kept at 28± 2° C and 70± 5% relative humidity and were fed with 5% (w/v) sucrose or fructose. Bemax Natural Wheatgerm (The Vitamins Company, England) plus lettuce leaves in Tehran or sea food (TetraMin) in Aberdeen were given to all larval stages.

Mosquitoes were narcotized by cold and dissected for their salivary glands and midguts. Dissected organs were collected and stored at -20° C until use. In sum 1840 pairs of salivary glands and 4150 midguts were used for immunizations and ELISA test.

Immunizing preparations Midguts were homogenized using Jenkons glass-glass homogenizer and centrifuged at 20,000g at 4° C for 15 min. Supernatant was removed and after deglycosylation (see below) was used as preparation No. 1 (MG-1). One ml of 1% triton X-100 and 1% tween20 in 0.5M tris- 0.15M saline buffer (pH= 7.2) was added to pellet and shook well. After 20 min at room temperature (rt), centrifugation was repeated and the supernatant and pellet were used as No. 2 (MG-2) and No. 3 (MG-3) preparations after deglycosylation.

Salivary glands were homogenized and centrifuged as before to obtain pellet (preparation No. 4 or SG-4) and supernatant (preparation No. 5 or SG-5) without deglycosylation.

Deglycosylation Pellet was suspended in 1ml cold 50 mM sodium acetate (pH= 4.5) and spun at 20,000g for 3min. Supernatant was discarded and pellet re-suspended in 0.5 ml of 10 mM periodic acid in sodium acetate and incubated at rt in dark for an hour, re-spun and pellet was re-suspended in sodium acetate. The suspension was spun again and 0.5 ml of 1% glycine in tris-saline buffer was added to pellet, incubated for 30 min at rt. The last spin was done and pellet was suspended in 0.5 ml tris-saline buffer.

As preparations No. 1 and 2 were soluble, to be deglycosylated, their proteins were precipitated by pure acetone between every step of deglycosylation.

Protein concentration assay Protein concentrations of preparations were measured using BCA Protein Assay kit (Pierce) and were adjusted with tris-saline buffer on 400 µg/ml of midgut and 120 µg/ml of salivary glands preparations.

Adjuvant Preparations were mixed in equal volumes with aluminum hydroxide gel (alhydrogel, Sigma) and kept for 2 h at rt or overnight at 4° C.

Immunizations Sixty female TO mice were divided in 6 equal groups. Groups 1-5 were immunized with one of the MG-1, MG-2, MG-3, SG-4 and SG-5 vaccines, respectively. Mice of group 6 (control group) received adjuvant plus tris-saline buffer. Four consecutive immunizations were performed with 3-4 week intervals. In every immunization, 100 µl of each vaccine was injected subcutaneously to shoulder of every mouse in relevant group.

Serology test Antigenic suspensions were prepared from midguts by the same procedure that had been used for vaccine preparations. Salivary glands were just homogenized to obtain antigenic suspension. Mice were bled by tail snaps before immunization and 10 d after 2nd, 3rd and 4th immunizations. Pooled plasma

sample from each mice group was prepared and classic microplate technique of ELISA, described by Sigma (16), was performed with midgut and salivary gland homogenates as antigens and anti-mouse IgG conjugated with HRP (Sigma) as secondary antibody.

Feeding Thirty to fifty female *An. stephensi* mosquitoes (5-10 d old) were collected in pots and kept starved overnight. Every mouse was anesthetized by peritoneally injecting 50 µl of anesthetic mixture (one volume of rompun and 2 volumes of ketamine) and offered to mosquitoes of a pot to feed.

Longevity assay Every day dead mosquitoes were collected and the number of fed ones was recorded. After 7 days live mosquitoes were counted and the overall number of fed mosquitoes was calculated. Daily survival, chance of survival, and cumulative chance of survival indices were calculated using below equations.

$$\text{Daily survival (DS)} = \frac{\text{Number of alive and fed mosquitoes in a day}}{\text{Number of alive and fed mosquitoes of the previous day}}$$

$$\text{Chance of survival (CS)} = 1 - \frac{\text{Number of died fed mosquitoes in a day}}{\text{Total number of fed mosquitoes of the pot}}$$

Cumulative Chance of survival (CCS) = CS of a day×CCS of the previous day (CCS of the feeding day (day 0) is 1.)

Fecundity A small dish containing a small piece of filter paper was put inside each pot before transferring mosquitoes to the pot, and two days after blood meal, 1 ml of water was added to this small dish. At the end of experiment filter papers were collected and the eggs laid on them were counted. By dividing number of total eggs laid in every pot to number of live mosquitoes of the same pot two days after feeding, mean number of eggs laid by one mosquito was computed.

Statistics EPI Info 6 software downloaded from Center for Diseases Control (CDC) site (http://www.cdc.gov/epiinfo/Epi6/ei6.htm), was used for entering data and Kruskal-Wallis test (equivalent to chi square).

Results

ELISA All pooled serums from each group of mice immunized with the antigens except group SG-4, showed increasing antibody titers from negative at the beginning of the study to at least 1:10,000 after 4th immunization with the antigen that had been used in appropriate vaccine preparation. Plasma samples from mice immunized with midgut antigens (MG-1, MG-2 and MG-3), showed cross-reactions with all midgut antigens (Table 1). Also cross-reaction was seen between mice group SG-5 and these antigens (MG-Ag1, MG-Ag2 and MG-Ag3). Antibody titer of group SG-4 after 4 immunizations did not exceed 1:1000. Also this group revealed no cross reactions with other antigens. No false-positive reaction was seen with control mice sera.

Longevity Mosquitoes fed on group MG-3 mice showed lowered life span but in rare instances this could reach significant level. Because of higher mean death rate of *Anopheles* mosquitoes at 3rd day after feeding on mice of this group (3.636 comparing with 1.273 of control group, $P< 0.01$), daily survival and chance of survival were significantly ($P< 0.05$) lower than control group at the same day (Table 2 and 3). Feeding on mice group MG-3 reduced mean cumulative chance of survival of mosquitoes when compared with control group (0.781 and 0.827, respectively, at 7th d after feeding), but this did not reach the significant level (Fig. 1).

Fecundity Every mosquito fed on control group mice, laid in average 131 (SD= 27.432) eggs. Mean number of eggs laid by *Anopheles* mosquitoes fed on mice group MG-3 was 100 (SD= 25.527) and significantly lower than control group ($P< 0.05$). All other mosquito groups produced fewer eggs (but not significant) than control group.

Table 1: Reciprocal antibody titers against midgut and salivary glands antigens in pooled serums of mice groups after forth immunizations

Groups \ Antigens	MG-Ag1	MG-Ag2	MG-Ag3	SG-Ag
MG1	10000	1000	1000	NEG.
MG2	10000	10000	10000	NEG.
MG3	10000	10000	10000	NEG.
SG4	NEG.	NEG.	NEG.	1000
SG5	10000	10000	10000	10000

Table 2: DS of mosquitoes fed on immunized and control mice during 7 days after blood feeding.

Mice group \ Days	1	2	3*	4	5	6	7
MG1	0.975	0.941	0.937	0.985	0.988	0.988	0.981
MG2	0.967	0.931	0.932	0.972	0.990	0.977	0.985
MG3	0.966	0.934	0.903*	0.982	0.972	0.977	0.966
SG4	0.973	0.950	0.977	0.986	0.985	0.978	0.978
SG5	0.971	0.937	0.969	0.979	0.995	0.972	0.983
Control	0.965	0.920	0.966*	0.993	0.985	0.991	0.970

*The difference between mean DS of MG3 and control group is significant ($P < 0.05$) on 3rd day according to Kruskal-Wallis One Way Analysis of Variance.

Table 3: CS of mosquitoes fed on immunized and control mice during 7 days after blood feeding

Mice group \ Days	1	2	3	4	5	6	7
MG1	0.975	0.943	0.942	0.988	0.990	0.990	0.985
MG2	0.967	0.936	0.942	0.978	0.993	0.981	0.990
MG3	0.966	0.937	0.917*	0.987	0.982	0.983	0.977
SG4	0.973	0.952	0.980	0.988	0.985	0.982	0.983
SG5	0.971	0.939	0.974	0.981	0.997	0.975	0.989
Control	0.965	0.922	0.971*	0.994	0.988	0.993	0.978

*Difference between mean CS of MG3 and control group is significant ($P < 0.05$) on 3rd day according to Kruskal-Wallis One Way Analysis of Variance.

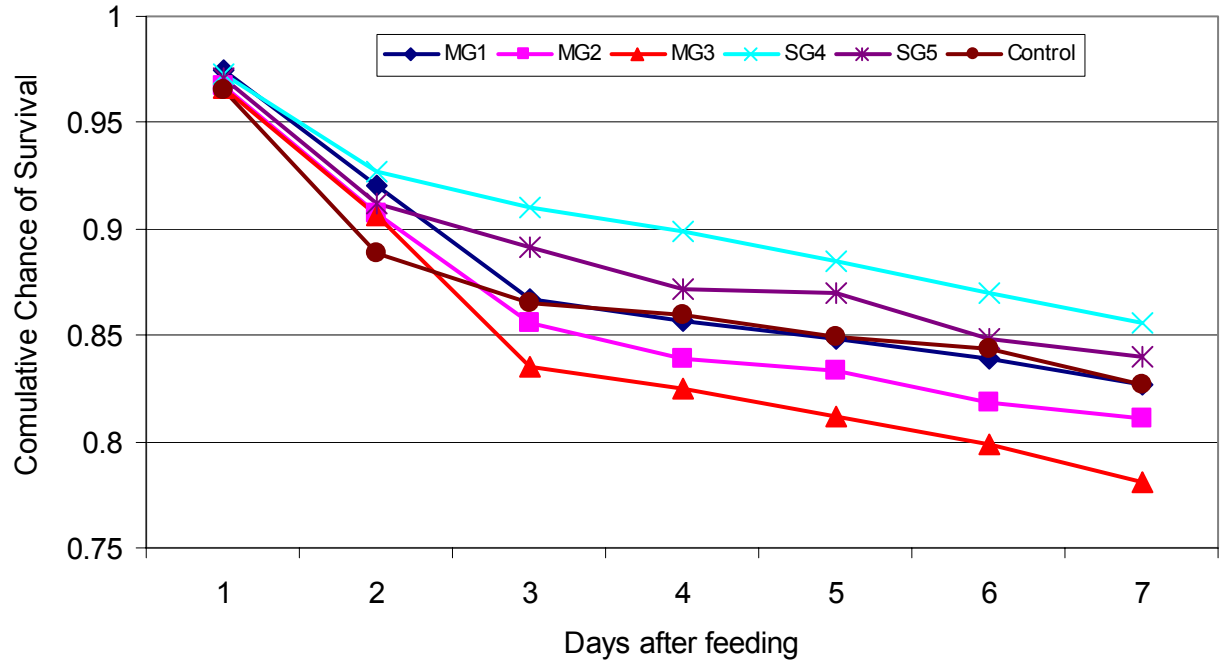

Fig. 1: CCS of *A. stephensi* mosquitoes fed on mice groups immunized with midgut (MG1, MG2 and MG3) or salivary gland antigens (SG4 and SG5) compared with control group during 7 d after feeding.

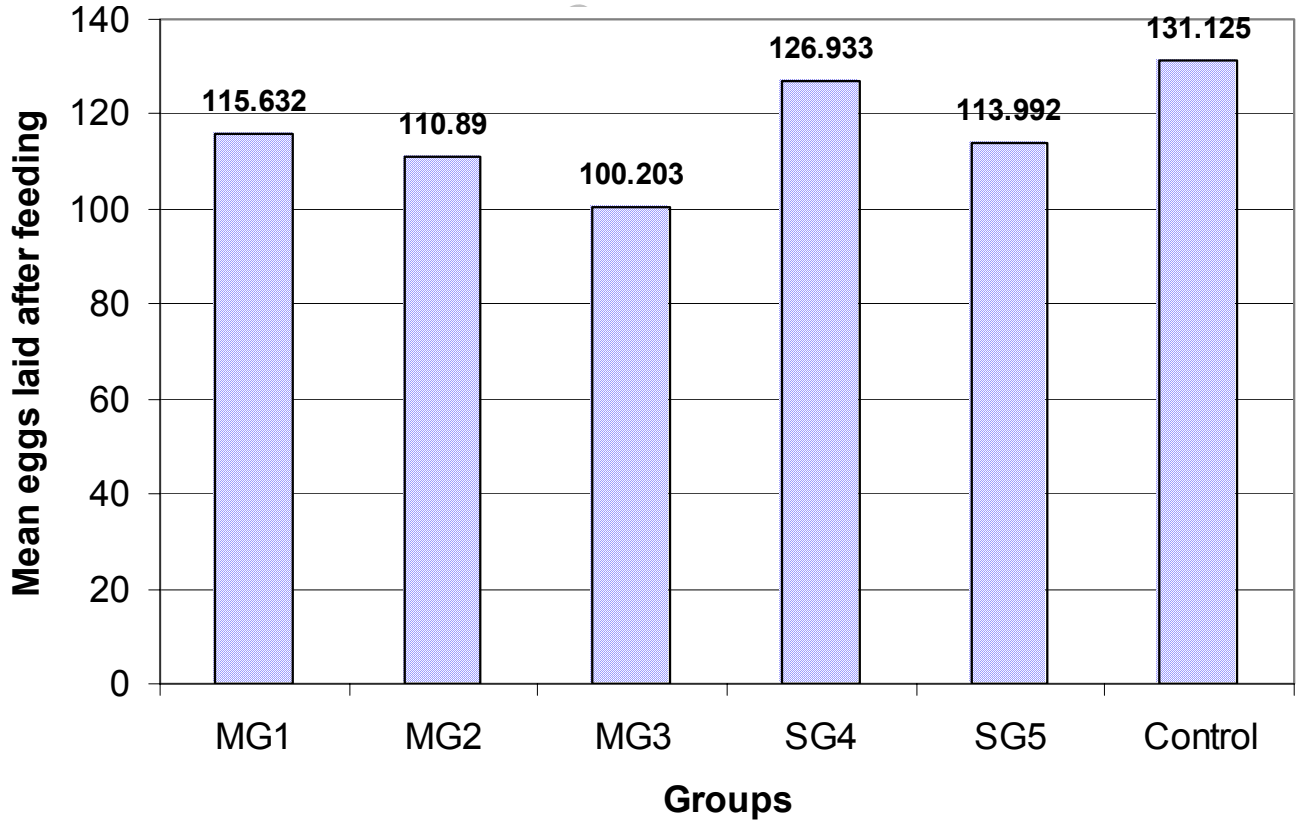

Fig. 2: Mean eggs number being laid by *Anopheles* mosquitoes fed on mice groups immunized with midgut (MG1, MG2 and MG3) or salivary gland antigens (SG4 and SG5) compared with control group.

Discussion

Confirmation of the success and economic viability of anti-tick vaccine introduced earlier (13) has raised the hopes of obtaining similar vaccines against malaria transmitting *Anopheles* mosquitoes. Alger and Cabrera (3) and Sutherland and Ewen (4) reported increased death rate and decreased fecundity of mosquitoes fed on animals immunized with mosquito antigens.

Others followed them by studying the effect of antibodies produced against antigens of mosquitoes on longevity (5, 7, 8), fecundity (5-9), and development of malaria parasites inside the vector (7-9). Midgut antigens are mostly considered as potential candidates to be used in anti-mosquito transmission blocking vaccines. This organ is not only the first one that comes in direct contact with antibodies of ingested blood, but also by passing through it the most susceptible stage of malaria life cycle, i.e. ookinete change to oocyst, takes place. All the other organs like salivary glands also should not be neglected, as it has been shown that IgG antibodies are capable to pass through midgut and reach mosquito's hemolymph, from which can distribute to all over the body (14). Despite of variability and sometimes opposite results, it is clear that all researchers share in belief that by finding suitable antigens and processing them in right way, producing an effective vaccine from mosquito antigens is feasible (3-9).

In this study, glycoproteins of midgut were deglycosylated before injecting to mammalian hosts and this is the first report of using deglycosylated antigens to immunize mammalian hosts against mosquitoes. Furthermore, as aluminum compounds are the only adjuvants used widely with human vaccines (15), for the first time, aluminum hydroxide gel was used as adjuvant to produce anti-mosquito vaccine.

Like most of previous studies, the best results in decreasing longevity and fecundity of mosquitoes were obtained from midgut antigens (6, 7, 9). MG-3 preparation made from insoluble antigens of midgut was able to significantly reduce the mean egg number laid by *A. stephensi* mosquitoes fed on immunized mice. These mosquitoes produced 23.5% fewer eggs when compared with control group. About the other preparations, reduction in fecundity (3-15%) was not significant.

Three days after feeding on mice immunized with MG-3, death rate of mosquitoes significantly increased, comparing with control group, but CCS did not reach significant point. Some researchers had similar results (4, 6, 9), but others could obtain significant levels (3, 5, 7, 8).

Three reasons may be considered for getting better results from MG-3. First, it may be because of MG-3s insoluble nature that means more stability in injection site and longer immune system stimulation. Second, acetone precipitation was applied on midgut soluble preparations (MG-1 & MG-2), during which, vast amount of proteins was lost. This may have resulted in lose of some proteins that otherwise might show significant results. Using other precipitation methods may increase titer and efficacy of produced antibodies against soluble preparations. Finally it is probable that some effective proteins in reducing fecundity or longevity are present in higher concentrations in insoluble or percipitable fraction of midgut (MG-3 in this study). For example, from basal lamina membrane of midgut that can be separated by as low gravities as 3000g, a 110 kDa protein band has been reported (10). Monoclonal antibody produced against this antigen (Mg25E), reduced both survivorship (significant in 7th d) and fecundity of *Anopheles* mosquitoes (7).

The effect of polyclonal antibodies against midgut and salivary glands antigens produced in immunized mice on the life cycle of *Plasmodium berghei* is under investigation and the results will be presented later.

Anti-mosquito transmission blocking vaccines having three simultaneous effects on malaria control, i.e. increasing mortality, decreasing fecundity, and reducing parasite development in vector, seem to be promising but continuous

studies are needed before introducing a successful vaccine.

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