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AN IMMUNOLOGICAL FACTOR THAT AFFECTS *ANOPHELES GAMBIAE* SURVIVAL[1]

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ABSTRACT. High titers of antibodies against *Anopheles gambiae* midguts were produced in New Zealand rabbits to identify midgut targets for an antimosquito vaccine. The serum from one of 8 rabbits (designated R2B6) killed 71.6% (Abbott's adjusted % mortality) of *An. gambiae* within 7 days. Mosquitoes ingesting R2B6 serum were unable to absorb their blood meal nutrients, resulting in reduced oviposition and egg hatching rates. *Anopheles stephensi* and *Anopheles arabiensis* were also killed when ingesting R2B6 serum but *Anopheles freeborni, Anopheles albimanus,* and *Aedes aegypti* were not affected. The mosquitocidal factor was a relatively large molecule (>100,000 MW) maintained at threshold levels in the sera and killing was complement independent. Mortality, however, was not IgG mediated, as determined by protein A-sepharose fractionation. This surprising finding confounds possibilities of using antibodies against whole mosquito midguts as a step in the development of antimosquito vaccines.

INTRODUCTION

Antimosquito vaccines, functioning to interrupt life-supporting processes in mosquitoes, could provide an innovative addition to traditional forms of mosquito control (Oaks et al. 1991). Several studies have reported a decrease in fecundity (Sutherland and Ewen 1974, Ramasamy et al. 1988, Srikrishnaraj et al. 1993) and survival (Alger and Cabrera 1972, Hatfield 1988) of mosquitoes ingesting antimosquito antibodies. No further studies have identified the mosquito antigens responsible for these biological effects. The purpose of this study was to produce antibodies against mosquito midguts to identify target proteins that could lead to the development of antimosquito vaccines.

MATERIALS AND METHODS

Mosquitoes: Laboratory colonies used in this study were: *Anopheles gambiae* Giles (G3), *Anopheles arabiensis* Patton (GMAL), *Anopheles stephensi* Liston (PAKISTAN), *Anopheles albimanus* Wied., *Anopheles freeborni* Aitken (MARYSVILLE), and *Aedes aegypti* (Linn.) (ROCKEFELLER).

Antigen preparation: Midguts from 100 newly emerged, unfed female *An. gambiae* adults were excised in chilled phosphate buffered saline (PBS), pH 7.4 (Dulbecco, Sigma, St. Louis, MO) and stored at −70°C in 100 µl of PBS. Prior to inoculation, midguts were homogenized in chilled PBS (pH 6.9) containing 2 mM phenylmethylsulfonylflouride (PMSF) (Sigma). Protein assays (Bio-Rad, Richmond, CA) on midgut homogenates indicated that each midgut contained *ca.* 2.5 µg total protein.

Animal immunization: Five male, 6-wk-old, New Zealand white rabbits received initial subcutaneous injections of 175 homogenized midguts mixed 1:1 in complete Freund's adjuvant (Sigma). Subsequent immunizations were delivered at 7–10-day intervals using the same concentration of crude midgut antigen mixed in incomplete Freund's adjuvant (Sigma). Two of the 5 rabbits (nos. 1 and 2) received one primary plus 6 booster immunizations. Three other rabbits (nos. 3, 4, 5) received one primary plus 9 booster immunizations. Following the same protocol described above for rabbits no. 1 and no. 2, 3 more rabbits (nos. 6, 7, 8) were each inoculated with 400 midguts. Sera were collected weekly and frozen at −70°C.

Immunoassays: Sera were tested for immunoreactivity against whole midgut homogenates using an enzyme-linked immunosorbent assay (ELISA). Fifty microliters of midgut homogenate in PBS (pH 7.4), equivalent to 2 µg/ml of protein, were added to each well of polyvinyl chloride microtiter plates (Dynatech Laboratories, Chantilly, VA) and incubated overnight at room temperature. Wells were aspirated, 100 µl of blocking buffer (0.5% boiled casein; Wirtz et al. 1989) was added to each well, and they were incubated for

[1] This study followed the Johns Hopkins Animal Care and Use Committee guidelines regarding the use of rabbits.

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Table 1. Immunological and mosquitocidal activity of sera from 8 rabbits immunized with *Anopheles gambiae* midgut homogenates. Standard deviation included with mean adjusted % mortality.

Rabbit no.	No. of boosts	IgG titer	No. of replicate cages	Mean adjusted % mortality[1,2]
1	6	1/64,000	8	2.8 ± 5.7
2	6	1/128,000	8	71.6 ± 7.6
3	9	1/64,000	3	3.1 ± 1.9
4	9	1/32,000	3	0.0 ± 0.0
5	9	1/256,000	3	1.6 ± 1.1
6	6	1/256,000	3	1.2 ± 1.7
7	6	1/256,000	3	4.5 ± 3.2
8	6	1/256,000	3	3.2 ± 4.5

[1] Mortality rates obtained on day 7.
[2] Mortality in immune-serum-fed group adjusted to mortality in control-fed group (Abbott 1925).

1 h (room temperature). After removal of blocking buffer, rabbit sera, diluted serially in blocking buffer (50 μl/well), were incubated for 2 h (room temperature). The wells were aspirated and washed 3 times with PBS plus 0.05% Tween 20 (PBS-TW). Peroxidase-labeled goat anti-rabbit IgG (H+L) (Kirkegaard & Perry, Gaithersburg, MD) diluted 1:2,000 in blocking buffer was added (50 μl/well) and incubated for 1 h (room temperature). Aspirated wells were then washed 3 times with PBS-TW and ABTS peroxidase substrate was added (100 μl/well) (Kirkegaard & Perry). IgM ELISAs followed the same protocol; however, peroxidase-labeled goat anti-rabbit IgM (μ chain specific) (Cappel, Durham, NC) was diluted 1:1,000 in blocking buffer. Absorbance values were read at 405 nm after 30 min. Sera taken before immunizations were used as negative controls (preimmune sera). All samples were tested in duplicate. The cutoff value determining ELISA endpoint titers was defined as the mean absorbance value of the negative controls + 3 standard deviations.

Bioassays: Mosquitocidal activity was determined by feeding sera to mosquitoes. Immune sera for each animal and boost were tested in parallel with mosquito feedings of preimmune sera taken from the same animal. Sera were mixed 1:1 with washed human erythrocytes and fed to 3–6-day-old mosquitoes via a membrane feeder at 37°C (Rutledge et al. 1964).

Engorged mosquitoes fed on each sera were transferred in equal numbers to 3–5 replicate cages (16–25 per cage). Mosquitoes in replicate cages were from the same cohort. Mosquitoes were maintained for up to 2 wk at 27°C, 85% RH, with an oviposition cup and a daily change of 3% Karo (Best Foods, Englewood Cliffs, NJ). Cages were checked daily and dead mosquitoes were counted and removed. Percent mortality in the immune-fed cages were corrected for normal mortality occurring in the preimmune-fed cages using Abbott's formula: ([negative control − test group]/negative control) × 100 (Abbott 1925).

The effect of immune sera on physiological parameters of mosquitoes was also determined. Engorged mosquitoes, sampled at various times after feeding, were examined for erythrocyte digestion (Vaughan et al. 1991) and ovarian status (World Health Organization 1975). Additionally, individual engorged females were monitored for oviposition. Eggs were counted and hatching rates determined.

Characterization of mosquitocidal sera: Serum (designated R2B6) was collected from rabbit no. 2, 1 wk after the 6th boost. To characterize the serum component responsible for mosquito mortality, the following experiments were done: dilution with preimmune serum, heat inactivation (56°C, 45 min), centrifugal microfiltration (Amicon, Beverly, CA), and protein A-sepharose purification of rabbit immunoglobulin G (IgG) (Sigma) (Harlow and Lane 1988). Protein A fractions were concentrated to 70% of their original volume by Speed-vac (Savant) and dialysed in 2 liters of PBS (pH 7.4) for 12 h at 4°C. Immunological and biological activities of the altered sera were determined as above. In addition, the mosquitocidal activity of R2B6 was examined by feeding the serum, mixed with erythrocytes (1:1), to *An. arabiensis, An. stephensi, An. albimanus, An. freeborni,* and *Aedes aegypti.*

RESULTS

Each of the 8 rabbits produced high IgG titers against *An. gambiae* midgut antigens, but only one rabbit produced serum with significant mosquitocidal activity (Table 1). The serum designated as R2B6 contained a relatively high concentration of antimidgut IgG (1:128,000). Interestingly, 16 samples taken from the 7 other rabbits had equally high or higher titers but had no effect on mortality; we therefore concentrated our efforts on the characterization of the R2B6 serum.

Upon ingestion of R2B6 by *An. gambiae,* significant mosquito mortality occurred from days 2 to 7 postingestion (Fig. 1). By day 7, 77 ± 14% of R2B6-fed mosquitoes were dead compared to 16 ± 13% of the control-fed group. Daily mortality in the R2B6-fed mosquitoes stabilized after day 7, becoming comparable to that of the control group.

Initially, R2B6-fed *An. gambiae* appeared to digest the blood meal normally because blood meal erythrocyte densities decreased over time

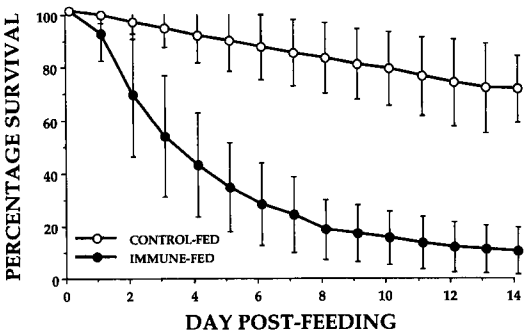

Fig. 1. Mean daily percent ± SD mortality of *Anopheles gambiae* mosquitoes after feeding on blood containing rabbit sera with antibodies against *An. gambiae* midgut antigens (R2B6; titer > 1:128,000) or preimmunized rabbit sera (control; titer < 1:50). Figure shows a composite of 12 individual feedings.

as in control-fed mosquitoes (ANOVA; [3 h: F = 4.09; df = 1,8; $P = 0.08$]; [24 h: F = 0.42; df = 1,8; $P = 0.53$]; [48 h: F = 1.09; df = 1,8; $P = 0.33$]). However, many mosquitoes in the R2B6-fed group began to die after 48 h (Fig. 1). Dead and moribund mosquitoes over the next several days appeared fully engorged. Instead of being small firm clots, blood meals consisted of dark liquid or gel with few intact erythrocytes. From days 3 to 6, ovarian development in 79% (23/29) of surviving R2B6-fed mosquitoes was arrested at the half-gravid stage and primary follicles were "locked" at stage III. Control-fed mosquitoes exhibited normal anopheline ovarian development. Follicles either grew and matured to stage V (14/24 = 58%) or reverted to stage IIa resting stage (10/24 = 42%). As a result, the oviposition rate in R2B6-fed mosquitoes (2/22 = 9%) was significantly reduced from that in control-fed mosquitoes (8/14 = 57%) ($\chi^2 = 7.60$, df = 1, $P = 0.006$). Among those mosquitoes that oviposited, there were no differences between R2B6-fed and control groups in the mean time to oviposition (3.5 and 4.0 days, respectively) (ANOVA; F = 0.20; df = 1,8; $P = 0.67$) or mean numbers of eggs laid (58 and 76 eggs, respectively) (ANOVA; F = 0.48; df = 1,8; $P = 0.52$). However, eggs from R2B6-fed females hatched one day later than eggs from control-fed mosquitoes.

Table 2. Mosquitocidal activity of anti-*Anopheles gambiae* midgut immune sera (boost no. 6) from rabbit no. 2 on different mosquito species. Standard deviation included with mean adjusted % mortality.

Species	No. of replicate cages	Mean adjusted % mortality[1,2]
Anopheles gambiae	8	72.1 ± 22.5
Anopheles arabiensis	2	100.0 ± 0.0
Anopheles stephensi	3	98.7 ± 2.3
Anopheles albimanus	3	6.8 ± 6.3
Anopheles freeborni	3	2.5 ± 2.4
Aedes aegypti	1	0.0 ± 0.0

[1] Mortality rates obtained after day 7.
[2] Mortality in immune-serum-fed group adjusted to mortality in control-fed group (Abbott 1925).

Table 3. Effect of different characterizations of anti-*Anopheles gambiae* midgut immune serum (boost no. 6) from rabbit no. 2 on *An. gambiae* survival. Standard deviation included with mean adjusted % mortality.

Serum treatment	No. of replicate cages	Mean adjusted % mortality[1,2]
Dilution		
Undiluted	3	54.8 ± 7.1
1:4	3	3.6 ± 5.2
1:8	3	0.9 ± 0.8
Heat inactivation		
Heat	6	72.0 ± 19.6
No heat	6	84.6 ± 4.8
Filtration		
>30,000 MW	3	80.1 ± 10.4
<30,000 MW	3	13.1 ± 14.7
>100,000 MW	2	96.8 ± 4.5
<100,000 MW	2	23.5 ± 5.1

[1] Mortality rates obtained after day 7.
[2] Mortality in immune-serum-fed group adjusted to mortality in control-fed group (Abbott 1925).

The R2B6 serum also had mosquitocidal activity against species other than *An. gambiae* (Table 2). However, the spectrum of activity was confined to *An. arabiensis* and *An. stephensi,* 2 species belonging to the same subgenus as *An. gambiae* (i.e., *Cellia*). Ingested R2B6 had no effect on more distantly related anopheline species (*An. albimanus* or *An. freeborni*) or on the non-anopheline, *Ae. aegypti.*

Several experiments were performed on R2B6 serum to characterize the serum components responsible for its mosquitocidal properties (Table 3). Activity was completely abolished when R2B6 was diluted 3-fold with preimmune serum. The killing factor was not filterable at 30,000 MW or 100,000 MW nor was it destroyed by heat-inactivation. The removal of total serum IgG by protein A-sepharose had no effect on the mosquitocidal activity of R2B6 (Table 4). Equivalent killing activity to native R2B6 serum was identified only in the IgG-depleted serum fraction, concentrated to 70% of its original volume. Rab-

Table 4. Effect of anti-*Anopheles gambiae* midgut immune serum (boost no. 6) from rabbit no. 2, fractionated by protein A-sepharose, on *An. gambiae* survival. Standard deviation included with mean adjusted % mortality.

Serum treatment	Experiment no.	No. of replicate cages	IgG titer	Mean adjusted % mortality[1,2]
Native		3	1/128,000	96.6 ± 2.4
IgG purified fraction	1	3	1/128,000	3.3 ± 4.7
	2	3	1/128,000	0.0 ± 0.0
IgG depleted fraction	1	3	1/100	95.2 ± 3.4
	2	3	1/100	80.0 ± 5.7

[1] Mortality rates obtained after day 7.
[2] Mortality in immune-serum-fed group adjusted to mortality in control-fed group (Abbott 1925).

bit no. 2 IgM levels peaked by boost 4 (titer: 1/400), decreasing to 1/200 by boost 6. R2B6 IgG-depleted fractions had IgM titers of 1/200.

DISCUSSION

This report demonstrates that antimosquito antibodies against whole anopheline midguts are of limited value for developing antimosquito vaccines. Of the 8 rabbits immunized, only one produced serum with significant mosquitocidal activity (R2B6). Although the other vaccinated rabbits produced serum antimidgut titers as high or higher than R2B6, none had mosquitocidal activity. Likewise, earlier studies reported high-titered antimosquito midgut IgG had minimal effect on mosquito mortality (Hatfield 1988, Ramasamy et al. 1988). This indicates that the concentration of antibody ingested does not determine mosquitocidal efficacy.

Mosquito mortality appeared to result from the inability of R2B6-fed mosquitoes to absorb nutrients from the blood meal because lysed erythrocytes formed a dark liquid or gel in the anopheline midguts. Engorgement and initial erythrocyte digestion appeared normal. Significant mortality began between 24 and 48 h after feeding. Most mosquitoes died fully bloated and surviving mosquitoes were unable to convert blood meal nutrients into egg production.

Initial characterization of the R2B6 mosquitocidal factor suggested that the mortality was antibody-mediated. The mosquitocidal activity was not filterable at 100,000 MW, indicating a large serum molecule was responsible for the mosquito mortality. The killing activity, however, was not recovered in the IgG-purified fraction after protein A separation even when concentrated to twice the IgG titer of native R2B6 serum from immunized rabbits (data not shown). Purified R2B6 IgG had immunological reactivity to midgut Ag, equivalent to native R2B6 sera, but the IgG fraction lacked the biological activity. Therefore, the mosquitocidal activity of R2B6 has so far not been identified to be IgG-mediated.

Remaining possibilities for the mosquitocidal effect include other large molecular weight serum factors (>100,000 MW) such as immunoglobulins or serum proteins. Such factors probably do not include the complement pathway because heat inactivation of R2B6 does not alter its ability to kill mosquitoes. Also, IgM does not appear to be a factor because higher titers were detected in R2 boost 4 serum that did not kill mosquitoes than in R2B6 serum and IgG-depleted R2B6 serum that killed mosquitoes. Although unable to isolate the factor(s) responsible for the mosquitocidal activity, it is obvious that some serum factor, however rare and irreproducible, has a critical role in mediating the biological activity.

Clearly, caution is necessary when using antibodies against whole mosquito midgut preparations in efforts to develop immunological mosquito control strategies. Although antimidgut antibodies provide a means to focus on particular midgut proteins, the results tend to be confusing and variable. Initiatives focusing on specific physiological or structural moieties may provide a better approach for making antimosquito vaccines.

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