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Antibody-Mediated Inhibition of *Aedes aegypti* Midgut Trypsins Blocks Sporogonic Development of *Plasmodium gallinaceum*

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The peritrophic matrix (PM) that forms around a blood meal is a potential barrier for *Plasmodium* development in mosquitoes. Previously, we have shown that to traverse the PM, *Plasmodium* ookinetes secrete a prochitinase and that an inhibitor of chitinase blocks further parasite development. Here we report that it is the mosquito trypsin that activates the *Plasmodium* prochitinase. Trypsin was identified as the chitinase-activating enzyme by two criteria: (i) trypsin activity and activating activity comigrated on one-dimensional gels, and (ii) activating activity and penetration of the PM by *Plasmodium* parasites were both hindered by trypsin-specific inhibitors. Subsequently, we examined the effect of antitrypsin antibodies on the parasite life cycle. Antibodies prepared against a recombinant blackfly trypsin effectively and specifically inhibited mosquito trypsin activity. Moreover, when incorporated into an infective blood meal, the antitrypsin antibodies blocked infectivity of *Aedes aegypti* mosquitoes by *Plasmodium gallinaceum*. This block of infectivity could be reversed by exogenously provided chitinase, strongly suggesting that the antibodies act by inhibiting prochitinase activation and not on the parasite itself. This work led to the identification of a mosquito antigen, i.e., midgut trypsin, as a novel target for blocking malaria transmission.

After ingestion by female mosquitoes, malaria parasites (gametocytes) emerge from erythrocytes in the mosquito midgut, where they are surrounded by a hostile environment rich in hydrolytic enzymes. To survive, the parasites must be resistant to a variety of digestive enzymes, including proteases (9). In this protease-rich milieu, malaria parasites undergo complex changes that include fertilization, differentiation into a motile ookinete, and about 1 day later, penetration of the midgut epithelium (reviewed in reference 14). At this time, the blood meal is completely surrounded by a thick chitin-containing peritrophic matrix (PM). Thus, the PM is a potential barrier for *Plasmodium* development in the mosquito. Previously, we have shown that inhibitors of chitinase block further development of the parasite (15) and that to traverse the PM, the ookinete secretes a prochitinase that can be activated by exogenous proteases (15). From these in vitro experiments, it became clear that the parasite is not able to fully activate its own chitinase; therefore, we hypothesized that the parasite relies on the protease-rich environment of the mosquito midgut to activate the chitinase before the penetration of the PM. In fact, we previously found that extracts of mosquito midgut 24 h after a blood meal can activate the parasite chitinase and that treatment of the midgut extract with diisopropylfluorophosphate, a serine protease inhibitor, abolished the chitinase-activating ability, suggesting the involvement of a serine protease in the activation process (15).

The mosquito digestive tract contains a complex set of endoproteases and exopeptidases (2). The major endoproteases are trypsins and chymotrypsins. A substantial amount of aminopeptidase activity is also present (3). Which of these proteases can activate the parasite chitinase was not clear. Here we identify mosquito midgut trypsin as the enzyme that activates *Plasmodium* chitinase. Moreover, we show that inhibition of trypsin activity by an antitrypsin antibody leads to a block of the *Plasmodium* life cycle in the mosquito. Thus, the mosquito trypsin is a novel vaccine candidate for blocking malaria transmission.

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MATERIALS AND METHODS

Parasites, mosquitoes, media, enzymes, and chemicals. *Plasmodium gallinaceum* (strain 8A) parasites were maintained in White Leghorn chickens by serial passage as described earlier (8). Ookinetes were prepared by incubating purified zygotes for 20 to 24 h in complete M199 medium (GIBCO) supplemented with 1 mM L-glutamine, streptomycin at 100 μg/ml, penicillin at 100 U/ml, and gentamycin at 125 μg/ml. The Liverpool/black eye strain of *Aedes aegypti* was raised at 26°C and 80% relative humidity and fed on sugar ad libitum. *Streptomyces griseus* chitinase was purchased from Sigma (catalog no. 1525). Endoproteinases Lys-C, Arg-C, Glu-C, Asp-N, and trypsin were purchased from Boehringer Mannheim (Indianapolis, Ind.).

Membrane feeding assay. Mosquitoes were fed blood through a stretched Parafilm membrane (American National Can, Greenwich, Conn.) as described by Sieber et al. (16). To assay transmission-blocking activity, antitrypsin sera or purified antibodies (see below) or leupeptin were fed with infected blood, and the mosquitoes were dissected 8 days after the blood meal. The midguts were stained for oocysts with 1.0% mercurochrome in water, and the number of oocysts per midgut was scored by light microscopy. Leupeptin (Boehringer Mannheim), dissolved in phosphate-buffered saline (PBS; pH 7.4) and mixed with freshly drawn *P. gallinaceum*-infected (15 to 30% parasitemia) chicken blood in heparin, was fed to overnight-starved *A. aegypti* mosquitoes. Sera from immunized mice were heat inactivated at 56°C for 30 min and mixed with infected blood at a ratio of 1:5, respectively. The mixture was fed to mosquitoes as described above. *S. griseus* chitinase (Sigma) was dissolved in PBS and added to the blood meal to a final concentration of 0.02 U/μl.

Chitinase activation assay. For activation of *P. gallinaceum* chitinase by fractionated *A. aegypti* midgut proteases, approximately 20 mosquito guts were dissected 24 to 30 h after blood feeding and homogenized in 1 ml of Na phosphate buffer (pH 6.8) on ice (15). The homogenate was centrifuged at 13,000 rpm for 30 min in a microcentrifuge (Eppendorf, Netheler+Hinz Gmbh, Hamburg, Germany) at 4°C. The resulting supernatant was lyophilized and resuspended in 500 μl of sodium dodecyl sulfate (SDS)-sample buffer without β-mercaptoethanol (0.125 M Tris-HCl, 20% glycerol, 2% SDS, 25 μg of bromophenol blue per ml [pH 6.8]) and incubated for 10 min at room temperature. The equivalent of 0.1 gut was fractionated in a gelatin-embedded 10% polyacrylamide gel and stained for protease activity (Zymogram; Novex, San Diego, Calif.). The remainder of

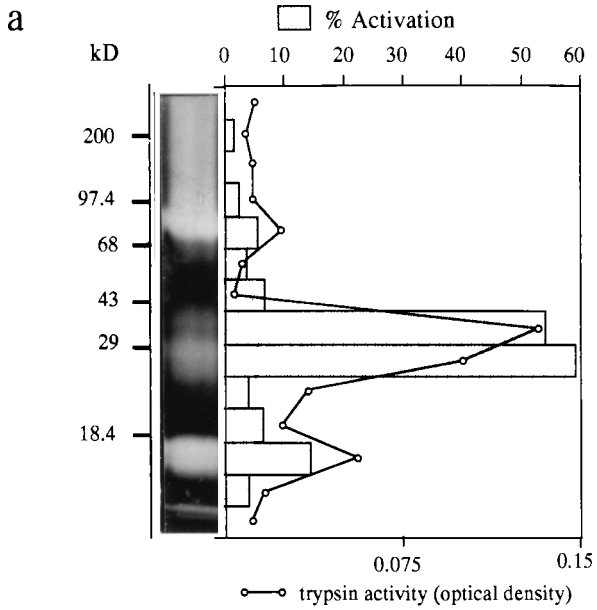
the sample was fractionated in a preparative 10% polyacrylamide–SDS gel (Novex), after which the gel was incubated twice for 10 min each time in 0.1% Triton X-100 in water to renature the enzyme and remove the SDS. The gel was cut into 5-mm slices; each slice was crushed, and the molecules embedded in it were eluted into 50 μl of Tris-HCl buffer (200 mM, pH 8.0) overnight on ice. An aliquot from each supernatant was assayed for trypsin activity with Chromozyme-TRY (Boehringer Mannheim) as described in the manufacturer's recommendations. Another aliquot was assayed for activation of *P. gallinaceum* chitinase as described before (15). For activation of chitinase, medium (10 ml) from a 24-h-old ookinete culture was concentrated to 0.5 ml with a Centriprep-10 concentrator (Amicon, Beverley, Mass.). Fifty microliters of the concentrated medium was mixed with 20 μl of protease supernatant and incubated at room temperature for 5 min. The reaction was stopped by the addition of 5 μl of diisopropylfluorophosphate (10 M final concentration). Chitinase activity was measured as described previously (6). Activation with specific endoproteinases was done by measuring the chitinase activity after incubation with endoproteinases Glu-C, Asp-N, Lys-C, and Arg-C (Boehringer Mannheim; final concentration, 10 ng/μl in 25 mM Tris-HCl–1 mM EDTA for 2 h at 37°C, pH 8.0), which cleave peptide bonds C-terminally to glutamic acid, N-terminally to aspartic acid, C-terminally to lysine, and C-terminally to arginine, respectively. Activities are expressed relative to the sample incubated with buffer alone.

Purification of antitrypsin-IgG. Three mice were vaccinated with recombinant *Simulium vittatum* trypsin-*Escherichia coli* TrpE fusion protein (mice 132, 133, and 134). Five mice were vaccinated with *E. coli* TrpE gene product (anthranilate synthase) alone (control 1, pooled sera from mice 135 and 139), and two mice were vaccinated with unrelated *E. coli* proteins migrating on gels with the same mobility as that of the recombinant fusion protein (control 2, pooled sera from mice 129 and 130). Coomassie blue-stained protein bands excised from a 10% polyacrylamide gel (at least 50 μg of proteins) were crushed and injected with RIBI adjuvant into each mouse (RIBI Immunochem Research Inc., Hamilton, Mont.). The first booster dose with a similar amount of protein with RIBI adjuvant was given 30 days after the primary immunization. The antibody titers of the immune sera were determined by enzyme-linked immunosorbant assay (ELISA) using the corresponding proteins as the capture antigen. After the second boosting (15 days after the first boosting), the corresponding antibodies reached a titer of 512,000. The chicken antibodies to the recombinant blackfly trypsin protein has been described previously (11). To purify IgG from mouse sera, 500 μl of serum from individual mice (immune group) or 500 μl of pooled sera (control 1 and control 2 groups) was diluted to 2 ml with 100 mM Tris-HCl [pH 8.0]. Two hundred microliters of packed protein A-Sepharose beads (Pharmacia, Uppsala, Sweden) equilibrated with 100 mM Tris-HCl (pH 8.0) was added to the diluted sera, and the mixture was incubated for 15 min at room temperature. The antibody-bound beads were used to make a column, washed first with 10 volumes of 100 mM HCl (pH 8) and then with 10 volumes of 10 mM Tris-HCl (pH 8). Antibodies were eluted with 100 mM glycine (pH 3) in batches of 500 μl in tubes containing 50 μl of 1 M Tris-HCl (pH 8) and mixed gently to neutralize. Fractions that contained most of the proteins were pooled and concentrated to 200 μl. Protein concentrations were measured by a bicinchoninic acid protein assay kit (Pierce, Rockford, Ill.).

Inhibition of mosquito midgut trypsin activity by antibodies against *S. vittatum* trypsin. Purified IgG (12, 1.2, 0.24, and 0.12 ng) from nonimmune (control 1 and control 2) and immune mice was added to midgut extract prepared from *A. aegypti* 24 h after a blood meal (0.01 midgut equivalent per assay). The mixtures were incubated at room temperature for 5 min and assayed for trypsin activity as described above.

RESULTS AND DISCUSSION

Mosquito midgut trypsin activates the *P. gallinaceum* chitinase. To investigate the mode of *Plasmodium* chitinase activation in the mosquito, blood-induced proteases from the *A. aegypti* midgut were fractionated by polyacrylamide gel electrophoresis (Fig. 1a). Each fraction was assayed for its ability to digest gelatin (Fig. 1a, left panel, photo), for its trypsin activity (right panel, line), and for its ability to activate *Plasmodium* chitinase (right panel, bars). A complex pattern of proteases in the size range of 14 kDa to more than 200 kDa was able to digest gelatin. In contrast, proteases with trypsin specificity migrated primarily with an M_r of 30 kDa, the characteristic size of mosquito (1, 7, 10) and blackfly (11) trypsins. Significantly, trypsin activity cofractionated with chitinase-activating activity (Fig. 1a), suggesting that mosquito midgut trypsin-like proteases are involved in the activation process. Trypsins are serine endoproteases that cleave proteins specifically at the C termini of lysine and arginine residues. If mosquito midgut trypsin is involved in activation of *Plasmodium* chitinase, it probably does so by cleaving at the C terminus of one or more



b

Treatment	none	Glu-C	Asp-N	Lys-C	Arg-C
Rel. activity	1	.89	.94	12.99	1.17

FIG. 1. (a) Activation of *P. gallinaceum* chitinase by fractionated *A. aegypti* midgut proteases. (Left panel) Extract of 0.1 mosquito gut equivalent was fractionated in a gelatin-embedded 10% polyacrylamide gel and developed for protease activity; (right panel, line) trypsin activity eluted from the corresponding regions of the gel shown in the left lane; (right panel, bar) percent activation of *P. gallinaceum* chitinase relative to the nonactivated enzyme. (b) Activation of the parasite chitinase by sequence-specific endoproteases (see text). Relative activity equals the activity of the protease-treated chitinase divided by the activity of untreated chitinase.

lysine and/or arginine residues. To investigate this possibility, we treated the secreted parasite chitinase with several specific endoproteinases that cleave proteins internally at specific sequences. In support of the assertion that mosquito midgut trypsin activates parasite chitinase is the finding that Lys-C but not Glu-C, Asp-N, or Arg-C was able to activate the chitinase (Fig. 1b) (15). Lys-C cleaves proteins at the C termini of lysine residues.

To further verify the hypothesis that mosquito trypsin is responsible for activation of the *Plasmodium* chitinase, we tested several structurally different trypsin inhibitors. Leupeptin was found to be a potent inhibitor of mosquito trypsin and did not interfere significantly with the survival of mosquitoes (13). When we fed mosquitoes with leupeptin (10 μg/μl) in a *P. gallinaceum*-infected chicken blood meal, parasite development to oocysts was completely blocked (Table 1).

Grotendorst and Carter (5) and Rosenberg et al. (12) also observed a reduction in the number of parasites that completed sporogony in mosquitoes fed infected blood containing trypsin inhibitors; however, they interpreted their results differently, suggesting that the block in parasite transmission was due to a failure of midgut proteases to inactivate blood complement. To determine whether this interpretation is correct or whether leupeptin acts solely at the level of penetration of the PM, we fed mosquitoes with a mixture of leupeptin and chitinase. Exogenous chitinase prevents PM formation (15). In the absence of a PM, leupeptin has no effect on the ability of

TABLE 1. Effect of the trypsin inhibitor leupeptin on the sporogonic development of *P. gallinaceum* in *A. aegypti* mosquitoes

Treatment[a]	Oocyst/gut[b]	Infectivity[c]	Infected/total[d]	*P*[e]
Leupeptin (10 μg/μl)	0	0	0/12	ND[f]
Leupeptin (5 μg/μl)	3.0 (0–11)	6.7	9/17	<0.001
Leupeptin (10 μg/μl) + chitinase	68.6 (1–103)	153.1	12/12	>0.1
Control (PBS)	44.8 (3–97)	100	15/15	

[a] *P. gallinaceum*-infected chicken blood was mixed with leupeptin or a mixture of leupeptin and chitinase and fed to the mosquitoes. The mosquitoes were dissected 8 days after the blood meal. The control group received no additives.
[b] Data are presented as William's geometric mean with ranges in parentheses.
[c] Infectivity is expressed as the percentage of mean oocysts per gut relative to that of the control group.
[d] Number of mosquitoes infected/total number of mosquitoes dissected.
[e] *P* values were determined by the Wilcoxon rank sum analysis by comparing the number of oocysts in mosquitoes in each group with that of the control group.
[f] ND, not determined.

ookinetes to penetrate the gut and develop into oocysts (Table 1). These data show that leupeptin affects sporogonic development only if the mosquito PM is intact. Thus, leupeptin probably acts by disrupting ookinete penetration of the PM and not by preventing inactivation of host blood complement.

Antitrypsin antibodies block transmission of malaria. The disruption of normal parasite development in mosquitoes by trypsin inhibitors prompted us to determine whether trypsin-specific antibodies can block mosquito trypsin activity and interfere with parasite development. Recently, we cloned a trypsin cDNA from the blackfly *S. vittatum* and determined that the sequence of the enzyme bears considerable homology with predicted trypsin sequences in general and with predicted mosquito trypsin sequences in particular (11). *Simulium* trypsin bears the highest sequence similarity with *Aedes* trypsins (1, 7, 11).

Antisera were raised against recombinant blackfly trypsin fusion protein. In preliminary experiments, we found that both control and immune antisera blocked trypsin activity. Many serum proteins, including antibodies that do not specifically recognize trypsin, can act as substrates for trypsin and thus are nonspecific competitive inhibitors. To minimize this nonspecific inhibitory effect, we purified the IgG fractions prior to assaying for inhibition of trypsin activity. Figure 2 shows that *Simulium* antitrypsin antibodies effectively inhibit *A. aegypti* midgut trypsin activity. At concentrations in which nonimmune IgG inhibited only about 10% of the trypsin activity, the immune IgGs inhibited trypsin activity by more than 90%, suggesting that the immune antibodies have a specific inhibitory effect. These findings and the preceding results suggested that antitrypsin antibodies might be used to interfere with the *Plasmodium* life cycle in the mosquito.

Antitrypsin antibodies were added to an infectious blood meal, and development of malaria parasites into oocysts was assessed. Antitrypsin sera inhibited the sporogonic development of *P. gallinaceum* in *A. aegypti* (Table 2). Control sera and antibodies to proteins of bacterial origin had no detectable effect. Addition of exogenous chitinase reversed the inhibition of *P. gallinaceum* development in *A. aegypti* (Table 2), again indicating that the antibodies did not directly affect parasite survival but instead blocked the parasite from crossing the PM in this mosquito. This observation is consistent with the hypothesis that mosquito protease is required to activate parasite-produced chitinase, which in turn is required for passage through the PM (Fig. 3).

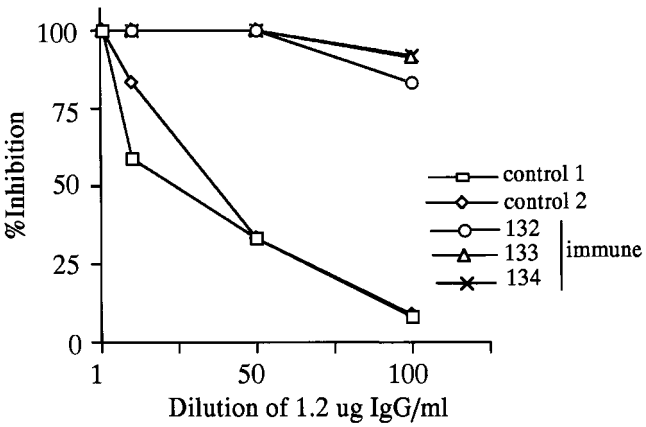


FIG. 2. Inhibition of mosquito midgut trypsin activity by antibodies against *S. vittatum* trypsin. Purified IgG (12, 1.2, 0.24, and 0.12 ng) from nonimmune (control 1 and control 2) and immune mice were added to a midgut extract prepared from *A. aegypti* 24 h after a blood meal. At high protein concentrations, the specific inhibitory effect of the antibody was masked by a nonspecific competition for the trypsin-active site by excess protein.

In principle, interference with blood digestion by the antitrypsin antibodies could lead to decreased egg production or even mosquito mortality. To address these issues, we measured the fertility and mortality of mosquitoes that were fed blood meals containing antitrypsin antibodies. Mosquitoes fed immune and control sera laid similar numbers of eggs and showed no significant difference in survival during the 9- to 10-day period of the experiment (Table 3). These observations may be explained by the following model: *Plasmodium* prochitinase secretion and activation occur during a narrow time window around the time of PM penetration by the ookinete. When prochitinase fails to be activated (e.g., because of the presence of antitrypsin antibodies), it diffuses away, thus preventing ookinete penetration. In turn, the antitrypsin antibodies are gradually destroyed by mosquito digestive enzymes, thus restoring blood digestion and egg production.

Model for ookinete penetration of the PM. Our data led to the following model (Fig. 3). At about 1 day after a blood meal,

TABLE 2. Effect of antitrypsin antibodies on sporogonic development of *P. gallinaceum* in *A. aegypti* mosquitoes[a]

Treatment[b]	Oocyst/gut	Infectivity	Infected/total	*P*
rBFtrypsin antiserum	0.16 (0–4)	3.5	3/14	<0.001
rBFtrypsin antiserum + chitinase	4.5 (0–21)	100	16/19	>0.1
rBFtrypsin antibody (purified IgG)	0.2 (0–5)	4.4	4/21	<0.001
rBFtrypsin antibody (purified IgG) + chitinase	1.9 (0–16)	42	14/21	>0.1
EcTrpE product antiserum (control 1)	4.1 (0–34)	91.1	15/19	>0.1
Unrelated *E. coli* protein antiserum (control 2)	4.6 (0–21)	102.2	15/18	>0.1
PBS (control 3)	4.5 (0–34)	100	17/19	

[a] See Table 1, footnotes *b* to *e*, for explanation of column heads.
[b] Sera and purified antibodies from immunized mice (see legend to Fig. 2) were heat inactivated and mixed with infected blood at a ratio of 1:5, respectively. The mixtures were fed to mosquitoes. *S. griseus* chitinase (0.02 U/μl) was added to the blood meal to disrupt PM formation. rBFtrypsin, recombinant blackfly midgut trypsin fused with *E. coli trpE* gene product; *EcTrpE*, *E. coli trpE* gene product; unrelated *E. coli* protein, *E. coli* protein of the same relative mobility as that of the recombinant fusion protein in an SDS-polyacrylamide gel.

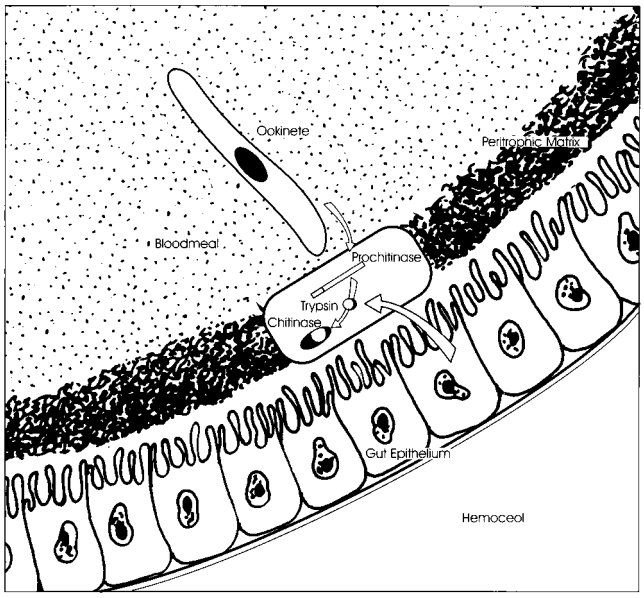


FIG. 3. Model for *Plasmodium* ookinete penetration of the mosquito PM. At 1 day after ingestion in a blood meal, gametocytes have completed their development into mature ookinetes. Ookinetes traverse the PM and then the gut epithelium and develop into oocysts on the side facing the hemocoel. To traverse the chitin-containing PM, the ookinete secretes a prochitinase. This enzyme is activated by a trypsin-like protease that is abundantly secreted by the midgut epithelium at the time of PM penetration. Chitinase activity induces local PM damage (16), facilitating penetration by the ookinete. Inhibition of protease activity by leupeptin or by antitrypsin antibodies prevents chitinase activation and blocks ookinete penetration.

Plasmodium zygotes have developed into motile ookinetes that move out of the blood bolus. Before invading the midgut epithelium, ookinetes encounter a rigid PM that surrounds the entire blood meal. On the basis of previous work (15) and on the present results, we propose that at this point parasites secrete a chitinase-like enzyme (6) that is not fully active but can bind tightly to pure chitin (15a). Concomitant with these events, the mosquito secretes large amounts of digestive enzymes, including trypsin. Immunocytological studies have shown that the trypsin diffuses through the porous PM (4, 11). Activation of the parasite prochitinase by the mosquito trypsin leads to the degradation of the chitin of the PM, causing local damage that allows the parasite to cross the PM (16).

An activatable chitinase may act as a timing device for the parasite. The presence of chitinase-activating trypsin in the midgut may act as an indicator that the condition outside the blood bolus is optimum. For example, proper timing in crossing the midgut epithelium may be critical for the parasite to satisfy its metabolic need during oocyst development and also to allow the parasite enough time to mature and acquire resistance against the degrading environment of the gut (3).

The development of an antimalarial vaccine is a goal of highest priority. To maximize the chances that a vaccine will succeed, it is desirable that it targets simultaneously the largest number of biological processes. The work presented here identifies the first candidate mosquito antigen that may be useful for blocking malaria transmission. Elucidating mechanisms that malaria parasites use to develop in the insect host may help researchers to design and test new strategies to control the spread of malaria parasites.

TABLE 3. Effect of antitrypsin antibodies on the fecundity and survival of *A. aegypti* mosquitoes[a]

Expt[b]	Fertility (eggs/mosquito)			% Survival		
	Control 1	Control 2	Immune	Control 1	Control 2	Immune
1	45.5	49.2	36.8	91.0	95.0	82.0
2	38.8	43.6	37.7	87.5	87.5	84.5
3	32.6	32.7	36.9	97.1	98.4	97.8

[a] Control and immune sera were fed to mosquitoes with chicken blood cells as described in Table 2, footnote *b*.

[b] In experiment 1, mouse anti-blackfly-trypsin serum (ELISA titer, 512,000) was used. Control 1, serum to *E. coli* TrpE gene product (ELISA titer, 256,000); control 2, serum to *E. coli* unrelated protein (ELISA titer, 512,000) (see Table 2, footnote *b*). In experiments 2 and 3, chicken anti-blackfly-trypsin serum (ELISA titer, 64,000) was used. The eggs laid per mosquito and the survival at day 9 or 10 are given. Differences between control and immune groups were not reproducible.

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