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Carbohydrate-mediated Recognition of Larval Mosquito Hosts by *Romanomermis culicivorax*[1]

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Abstract: Proteases, glycosidases, and lectins were tested and the results supported a role in host recognition for glycoproteins containing β-glucose and α-mannose on the cuticular surface of host and parasite. Carbohydrates containing α-glucose, galactose, fucose, or N-acetylglucosamine residues apparently are not involved in nematode attachment. Chitin or a related N-acetylglucosamine polymer was found in *R. culicivorax* preparasites. Treatment of preparasites with neuraminidase, which hydrolyzes sialic acids, increased nematode attachment to *Anopheles freeborni* larvae.

Key words: Anopheles freeborni, carbohydrate, host recognition, mosquito cuticle, nematode cuticle, *Romanomermis culicivorax.*

Regulation of host recognition, membrane-mediated morphogenesis, and intercellular communication by glycoproteins associated with cell walls and membranes has been documented in many biological systems (8,14,17,29,36). Recently carbohydrates and glycoproteins have been implicated in the interaction of nematodes with nematophagous fungi (19–22,31), in nematode chemotaxis (20,22), and in related nematode behavior involved in host–prey recognition (41). Previous emphasis on nematode chemoreception has been on plant-parasitic or saprophytic species.

Romanomermis culicivorax is an obligate parasite of mosquito larvae. It has strict requirements for growth and maturation into the postparasitic stage (6,10,11). Preparasites must recognize larval hosts which they actively seek, and upon contact the nematodes attach to the host cuticle, penetrate into the hemocoel, and complete parasitic development in 6–10 days. The initial interaction of host and parasite involves at least six separate steps: short range larval detection, reorientation to the host, attachment, search-boring, momentary host paralysis, and cuticle penetration (37).

Romanomermis culicivorax preferentially parasitizes *Anopheles* spp., but it also parasitizes at least 12 other genera of mosquitoes (32,33). Preparasites also penetrate several species of simuliids if they are exposed to very high densities of the nematode (12).

Preparasites react to the proximity of host larvae by secreting a substance that binds to concanavalin A and may function as a glue to aid in binding the nematode to mosquito cuticle (37, Platzer, pers. comm.). Infection is initiated by short-range interactions or direct contact with a susceptible host and presumably involves surface-mediated recognition by the nematode of specific chemical signals originating from the mosquito cuticle. Evidence of a role for glycoproteins containing β-glucose and α-mannose in this recognition process is presented here. The presence of chitin or a related N-acetylglucosamine polymer, rarely found in dauer stages of nematodes, also is reported from *R. culicivorax* preparasites.

MATERIALS AND METHODS

Source and maintenance of organisms: Romanomermis culicivorax preparasites were obtained by flooding sand cultures containing eggs and postparasites with distilled water several hours before initiation of experiments. Sand cultures were provided by S. Critchfield, Sacramento Yolo Mosquito Abatement District. The nematodes were reared in vivo in *Culex pipiens* larvae using standard protocols (7).

Newly emerged preparasitic juveniles

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were obtained by filtration through 60-μm-pore nylon screen and concentrated onto filter paper with a gentle vacuum. Preparasites were then suspended in pH 6.5 distilled water containing 5 mM calcium and 1.3 mM manganese (CaMn) to give a final density of 200–300 preparasites/ml. This solution was chosen because several of the lectins used in these evaluations required calcium and (or) manganese for optimum binding activity. For some of the enzyme treatments described here, preparasites were suspended in solutions in which optimal enzyme activity occurred.

Mosquito larvae used in the bioassay were obtained from a laboratory colony of *Anopheles freeborni* established from mosquitoes collected in Sutter and Yuba counties, California. Second-instar or early third-instar *A. freeborni* larvae were used in all assays. Host larvae were killed by immersion for 30 seconds in water heated to 50–60 C prior to the assays to eliminate any behavior-mediated variation in nematode host recognition. For a given set of experiments, larvae were of approximately the same size and free from various protozoa which occasionally inhabit the cuticle surface of colony mosquitoes.

Lectin incubations and bioassays: Preparasite density was adjusted to 200–300 nematodes/ml. Initial evaluations involved preincubation of 5 or 10 ml of preparasite suspension in CaMn with 25 or 75 μg lectin/ml for 1 hour. Lectin (Sigma Chemical Company, St. Louis, MO) solutions were filtered and the preparasites were resuspended in CaMn following extensive washing in distilled water. In a second set of experiments, host larvae were preincubated with the various lectins. Ten heat-killed *A. freeborni* larvae were exposed to 25 or 75 μg lectin/ml in CaMn for 1 hour, filtered, washed, and resuspended in 1 ml preparasite suspension. Each experiment was repeated 3–6 times.

Bioassays were run in 1 ml CaMn in a 1.6-cm-d well of a polystyrene tissue culture plate (Becton Dickinson, Rutherford, NJ). In the limited area of the plastic wells, the position of the larvae affected the number of nematode attachments because of differential distribution of the preparasites. Larvae near well bottoms or edges were generally exposed to greater numbers of nematodes. To reduce variability, the number of larvae in these positions was kept constant during a given series of bioassays. Assays involved exposing larvae to nematodes for 5 minutes following the lectin incubations and counting the preparasites firmly attached to each of the 10 larvae. Controls with comparable incubation, filtration, and washing protocols in the absence of lectin were run for each assay.

Enzyme treatments: Bioassays were conducted following treatment of preparasites or mosquito larvae with various enzymes. Relatively high concentrations of glycosidases were used because insect cuticle is refractile to enzymolysis (26,27). Controls for each treatment used identical incubation times, temperatures, and buffers without the added enzyme.

Nematode treatments were as follows: trypsin, EC 3.4.21.4—incubation for 1 hour in 150 units enzyme in 1.5 ml CaMn, pH 6.8; neuraminidase, EC 3.2.1.18—incubation for 1 hour in 10.5 units enzyme in 1.5 ml CaMn, pH 5.5 at 35 C; chitinase, EC 3.2.1.14—incubation for 3 hours in 36 units enzyme in 3.0 ml 0.02 M Na phosphate, pH 6.2 at 23 C.

Mosquito larvae were treated as follows: trypsin, EC 3.4.21.4—incubation for 1 hour in 300 units enzyme in 3.0 ml 0.1 M Na phosphate, pH 7.1 at 23 C; β-glucosidase, EC 3.2.1.21—incubation for 90 minutes in 150 units enzyme in 3.0 ml 0.1 M Na acetate, pH 5.0 at 35 C; α-glucosidase, EC 3.2.1.20—incubation for 3 hours in 150 units enzyme in 3.0 ml 0.1 M Na phosphate, pH 6.0 at 35 C; β-galactosidase, EC 3.2.1.23—incubation for 90 minutes in 150 units enzyme in 3.0 ml 0.1 M Na phosphate, pH 7.1 at 35 C; α-mannosidase, EC 3.2.1.24—incubation for 3 hours in 21 units enzyme in 3.0 ml 0.1 M Na acetate plus 0.1 mM Zn, pH 4.5 at 35 C. Enzymes

TABLE 1. Number of preparasites attached to *Anopheles freeborni* after incubation of nematodes with lectins in each of six trials.

Source of lectin†	1	2	3	4	5	6
Control	3.4 ± 1.8	3.4 ± 1.7	6.8 ± 4.2	7.1 ± 2.4	4.8 ± 2.5	5.5 ± 4.4
Canavalia ensiformis (jackbean)	0.9 ± 1.4*	1.4 ± 0.7	—	2.0 ± 1.6* 2.4 ± 2.5*	—	—
Lens culinaris (lentil)	0.8 ± 1.6	—	2.5 ± 2.2*	2.5 ± 2.2*	—	—
Triticum vulgaris (wheat germ)	4.1 ± 4.2	—	2.1 ± 1.4*	5.1 ± 3.7	2.7 ± 1.6 2.5 ± 2.4	—
Arachis hypogaea (peanut)	3.0 ± 2.4	2.9 ± 1.7 3.3 ± 1.2	—	—	—	—
Lotus tetragonolobus (pea)	2.5 ± 1.4	—	4.8 ± 1.9	—	4.9 ± 1.2	—
Limulus polyphemus (crab)	—	—	—	—	—	1.8 ± 1.8 3.4 ± 1.8 3.2 ± 3.0

Mean ± standard deviation for 10 host larvae after 5 minutes exposure to nematode suspensions. —: Experiment was not done during this trial.
* = Significantly different from control at $\alpha = 0.05$.
† All lectins used at a concentration of 75 μg/ml.

were obtained from Sigma Chemical Company.

Statistical analyses: Significance of the data was evaluated by comparison of the various treatments with the appropriate control assay using attachment to individual larvae as a single data point, i.e., 10 replications per trial using a given treatment. Each treatment was repeated at least three times. Statistical significance at $\alpha = 0.05$ was calculated with Duncan's multiple-range test using SAS programs.

RESULTS

Nematode cuticular carbohydrates: Incubation of *R. culicivorax* preparasites in jackbean (concanavalin A) and lentil lectins resulted in significant reductions in subsequent nematode attachment to *A. freeborni* larvae (Table 1). The major affinity of these two lectins is for glucose and mannose residues, although they will bind N-acetyl-D-glucosamine to a lesser extent. Pea and peanut lectins, with major affini-

TABLE 2. Number of preparasites attached to *Anopheles freeborni* after incubation of mosquito larvae with lectins in each of six trials.

Source of lectin†	1	2	3	4	5	6	7
Control	4.8 ± 2.5	6.4 ± 3.5	5.5 ± 5.4	3.8 ± 2.4	5.3 ± 3.3	3.9 ± 2.3	2.9 ± 2.3
Jackbean/25	2.8 ± 1.5	2.3 ± 2.0*	—	1.6 ± 0.7*	—	—	—
Jackbean/75	1.0 ± 0.8*	0.8 ± 0.6*	—	1.2 ± 1.1* 0.9 ± 1.2*	—	—	—
Lentil	—	—	2.4 ± 2.3 2.6 ± 2.1	1.1 ± 1.6*	1.1 ± 1.3* 1.2 ± 1.2*	—	—
Wheat germ	—	—	1.7 ± 1.8*	—	4.9 ± 3.2 1.7 ± 1.1	1.7 ± 1.1	2.3 ± 2.5 1.5 ± 1.7 1.2 ± 1.0
Peanut	—	—	—	3.1 ± 1.6 2.8 ± 2.2	5.4 ± 2.2	—	—

Mean ± standard deviation for 10 host larvae after 5 minutes exposure to nematode suspensions. —: Experiment was not done during this trial.
* = Significantly different from control at $\alpha = 0.05$.
† All lectins used 75 μg/ml except for jackbean/25 which corresponds to 25 μg/ml of the lectin.

TABLE 3. Number of preparasitic nematodes attached to each *Anopheles freeborni* larva treated with glycosidase or trypsin in each of nine trials.

Treatment	1	2	3	4	5	6	7	8	9
Control	6.5 ± 2.6	5.5 ± 2.9	4.3 ± 1.8	9.0 ± 4.3	5.2 ± 2.9	6.0 ± 2.4	3.4 ± 2.0	3.1 ± 2.0	5.0 ± 2.0
β-glucosidase	2.4 ± 1.6* 1.9 ± 1.6*	— —	— —	6.1 ± 4.1 6.1 ± 4.2	—	—	1.1 ± 1.2*	—	—
α-mannosidase	—	—	—	—	1.7 ± 1.6 2.6 ± 1.9	—	1.3 ± 0.9* 0.4 ± 1.0* 1.3 ± 1.0*	—	—
β-galactosidase	—	—	4.4 ± 3.3 4.7 ± 2.8	8.7 ± 3.7	—	—	—	—	—
α-glucosidase	—	4.0 ± 2.2 5.7 ± 3.0	—	—	—	4.9 ± 3.0 6.5 ± 2.0	—	—	—
Trypsin	—	—	—	—	—	—	—	1.0 ± 1.1* 0.1 ± 0.3* 0.7 ± 0.7* 0.7 ± 0.8*	—
Neuraminidase	—	—	—	—	—	—	—	—	5.8 ± 3.8 4.5 ± 2.7 4.4 ± 2.7

Incubation conditions and concentrations listed in Materials and Methods. Controls were run in identical buffers and temperatures without the enzymes.
Mean ± standard deviation for 10 host larvae after 5 minutes exposure to nematode suspensions. —: Experiment was not done during this trial.
* = Significantly different from control at $\alpha = 0.05$.

TABLE 4. Number of preparasitic nematodes treated with enzymes that attached to *Anopheles freeborni* larvae in each of four trials.

Treatment†	1	2	3	4
Control	4.9 ± 3.3	7.9 ± 2.3	5.0 ± 2.0	5.2 ± 1.7
Neuraminidase	10.7 ± 10.8*	—	9.1 ± 4.1* 9.8 ± 4.9*	—
Chitinase	—	3.7 ± 2.0 4.7 ± 4.9 5.5 ± 3.3	—	—
Trypsin	—	—	—	1.2 ± 1.2* 0.7 ± 1.3 0.9 ± 1.1*

Mean ± standard deviation for 10 host larvae after 5 minutes exposure to nematode suspensions. —: Experiment was not done during this trial.
* = Significantly different from control at $\alpha = 0.05$.
† All lectins used at a concentration of 75 μg/ml.

ties for L-fucose and galactose, respectively, had no effect on host recognition by the nematode (Table 1).

Erratic results were obtained following incubation with wheat germ agglutinin and limulin (Table 1), which bind polymeric N-acetylglucosamine and sialic acids, respectively. This suggests indirect interference due to the presence of bulky lectin proteins on the nematode cuticle.

Anopheles freeborni cuticular carbohydrates: Concanavalin A and lentil lectin selectively interfered with nematode attachment when mosquito larvae were incubated with these proteins (Table 2). Wheat germ agglutinin apparently bound to larval chitin and erratically interfered with the host recognition response. As in the case of preparasite treatments, this was interpreted as an indirect effect on the host–parasite interactions.

Effect of enzymic modifications on nematode and larval cuticle: Confirmation of the nature of the cuticular components which mediate host recognition was obtained using specific glycolytic enzymes and trypsin. Enzyme treatments indicated that both α-mannose and β-glucose were components of complex carbohydrates involved in the choice of a potential host by *R. culicivorax* preparasites (Table 3). Trypsin digestion further suggested that it is a glycoprotein on the larval mosquito cuticle which is involved in this interaction (Table 3).

Related experiments using nematodes were restricted because of the sensitivity of the preparasites to a number of the buffers needed to optimize enzyme activity. Attachment of preparasites to larvae after incubation with neuraminidase, which acts on sialic acids, was significantly enhanced (Table 4). Erratic attachment following chitinase treatment indirectly confirmed the presence of polymeric N-acetylglucosamine (Table 4).

DISCUSSION

Recent work by Bird (4) has emphasized the presence of a thin glycocalyx covering the cuticles of some nematode species. Frequent reference is made in this report to nematode cuticle-mediated host recognition. Our contention of the involvement of cuticular rather than glycocalyx receptors is based on the absence of a glycocalyx on *R. culicivorax* preparasites documented by Platzer and colleagues (Platzer, pers. comm.).

The approach taken in this investigation has its limitations. For instance, it is known that concanavalin A is usually present as a tetramer in solution, and treatment of the host or parasite can possibly increase nematode attachment because of cross reaction of sugar residues on their respective cuticles with single concanavalin A molecules. This is very unlikely, however, since what usually occurs is saturation of all four binding sites of a given lectin by carbo-

hydrates on the substrate surface (16,24). A simple signal-receptor interaction probably would not be sufficient to attach a nematode to a mosquito larva. Attachment apparently involves active secretion by the nematode of a concanavalin A-positive material which may promote adhesion (37, Platzer, pers. comm.). The basic conclusions of this paper are further supported by the lentil lectin (a dimer) experiments and the corresponding enzymic modification experiments. Also, fluorescently labelled concanavalin A binds only to the cephalic region and the amphids of preparasites (Platzer, pers. comm.), suggesting localization of carbohydrate-containing molecules involved in host recognition.

Host seeking, recognition, and penetration by *R. culicivorax* involves at least six steps (37). The assays described here involve the first three, i.e., short-range host seeking, reorientation to host, and attachment. This last step occurs concomitantly with secretion from the mouth of a concanavalin A-positive material (37, Platzer, pers. comm.), which may be involved in nematode attachment to mosquito larvae. Lectin and enzymic treatments could disrupt one or all of these discrete steps in the invasion process.

Chemical composition (5,18,28) and related physical and ultrastructural properties (1,15) of terrestrial insect cuticle has been studied extensively; however, very little has been published on physical and chemical characteristics of aquatic insect cuticle. While the epicuticle invariably consists of a well-developed lipid layer in terrestrial insects (1,15), this is not necessarily the case in aquatic insects where protection from desiccation is not a major problem (2). Glycoproteins rather than lipids on the mosquito cuticular surface appear to signal the presence of compatible host larvae. Carbohydrates probably are also involved in mosquito larval host recognition by two entomopathogenic fungi, *Coelomomyces psorophorae* (Chytridiomycetes: Blastocladiales) (25) and *Lagenidium giganteum* (Oomycetes: Lagenidiales) (unpubl.).

Sialic acids have been characterized histochemically on the surface of nematodes (38,40) and are involved in many of the nematode recognition phenomena which have been investigated (22,41). Sialic acids do not appear to be involved in host recognition by *R. culicivorax* preparasites, since there was no consistent reduction in parasite attachment after incubation with limulin; however, neuraminidase treatment of preparasites increased their rate of attachment to mosquito larvae. It is difficult to envision the basis for enhanced host recognition by removal of sialic acids. Enhanced cell adhesion has been noted following treatment with exoneuraminidases and endoneuraminidases in other biological systems (35).

Chitinase treatment of *R. culicivorax* preparasites reduced attachment to mosquito cuticle. Preincubation with wheat germ agglutinin, which binds very selectively to dimers or higher polymers of N-acetylglucosamine (the monomeric subunit of chitin) (13,30), resulted in erratic nematode attachment. Such behavior unexpectedly suggests that chitin or a short chain polymer of N-acetylglucosamine occurs on the surface of preparasites. Chitin has been found in the eggs of a restricted number of species in the Nematoda (3), and a dimer or higher polymer of N-acetylglucosamine was documented using wheat germ agglutinin binding to larvae of the ryegrass nematode, *Anguina agrostis* (39) and to two species of *Meloidogyne* (9). Further characterization of the chemical nature of the carbohydrates and glycoproteins involved in this host–parasite interaction is in progress.

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