ULTRASTRUCTURE OF THE IMMUNE RESPONSES OF *ANOPHELES QUADRIMACULATUS* TO *ROMANOMERMIS CULICIVORAX* (NEMATODA: MERMITHIDAE) INFECTION

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ABSTRACT

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The ultrastructure of the early immune response of *Anopheles quadrimaculatus* larvae to invasion by the mermithid nematode *Romanomermis culicivorax* was examined in experimental infections. Host hemocyte recognition of *R. culicivorax* began immediately after infection. Cellular encapsulation and premelanotic reactions were noted. The proportions of plasmatocytes and especially granulocytes increased with hemocytes or their products adhering to invading nematodes within minutes of infection. Degranulation of granulocytes occurred when the host recognized the nematode surface, although contact between the hemocyte and the parasite was not required for recognition. Concurrently, the parasitic nematodes shed a dispensable sheet-like surface coat that intermittently interrupted the encapsulation process by serving as a physical barrier against the host immune system.

Key words: Culicidae, granulocyte, hemocyte, host-parasite relationship, mosquito, nematode, plasmatocyte, prohemocyte, surface coat, transmission electron microscopy.

RESUMEN

Shamseldean, M. M., E. G. Platzer, y R. Gaugler. 2006. Ultraestructura de la Respuesta Inmune de *Anopheles quadrimaculatus* a la Infección por *Romanomermis culicivorax* (Nematoda: Mermithidae). Nematropica 36:243-249.

Se examinó la ultraestructura de la respuesta inmune temprana de larvas de *Anopheles quadrimaculatus* a la invasión por el nematodo mermítido *Romanomermis culicivorax* en infecciones experimentales. El reconocimiento de *R. culicivorax* por parte de los hemocitos del hospedante se inició inmediatamente después de la infección. Se observaron reacciones premelanóticas y encapsulación celular. La proporción de plasmatocitos y granulocitos aumentó con la adhesión de hemocitos o sus productos a los nematodos invasores durante los primeros minutos después de la infección. Se observó degranulación de granulocitos cuando el hospedante reconoció la superficie del nematodo, aunque no se requirió contacto entre el hemocito y el parásito para el reconocimiento. De manera concurrente, los nematodos parásitos produjeron una cubierta superficial que interrumpía intermitentemente el proceso de encapsulación, actuando como una barrera física contra el sistema inmune del hospedante.

Palabras clave: Culicidae, granulocito, hemocito, relación hospedante-parásito, mosquito, nematodo, plasmatocito, prohemocito, cubierta superficial, microscopía electrónica de transmisión.

INTRODUCTION

Mermithid nematodes are obligate parasites of insects, spiders, crustaceans, earthworms, leeches and mollusks (Poinar, 1979). They tend to be specific to a single host species or family. Most members have seen little study with the notable

exception of species parasitizing mosquitoes, particularly *Romanomermis culicivorax* (Ross and Smith). The infective stage of this species is the preparasite, which hatches from the egg and swims to find hosts. Once contact with a suitable mosquito larva is made the stylet is wielded to pierce the cuticle and permit parasite entry. The parasitic stage grows slowly for the first three to four days before rapidly increasing in size over the following three to four days and development is complete. The parasitic stage then molts to the postparasitic stage and makes an emergence wound which is fatal to the host. After emergence, postparasites of *R. culicivorax* burrow into the soil at the bottom of the mosquito pool, mate, and lay eggs to complete the life cycle.

Romanomermis culicivorax has been intensely investigated with aspirations of using the parasite for biological control (Platzer *et al.,* 2005), an interest fueled substantially by its broad host range among culicines. Over 90 species of mosquitoes in 13 genera are susceptible to *R. culicivorax* infection (Petersen, 1985; Peng *et al.*, 1992). The basis for this broad host range is unclear. Encapsulation is the main immune response to metazoan parasites, and both cellular and humoral encapsulation of mermithids has been reported in mosquitoes (Poinar, 1979; Poinar *et al.*, 1979). Yet few mosquito species are able to inhibit the development of *R. culicivorax* by this means (Petersen, 1985). Hall *et al.* (1975) reported that active suppression of the encapsulation reaction by *R. culicivorax* appeared unlikely. Ultrastructural studies of the host immune response to *R. culicivorax* invasion can assist our understanding of mermithid host specificity. We followed the early parasitic phase of *R. culicivorax* in the permissive host *Anopheles quadrimaculatus* (Say) with transmission electron microscopy (TEM).

MATERIALS AND METHODS

Romanomermis culicivorax was maintained on an autogenous strain of *Culex pipiens* (L.) according to the procedures of Platzer and Stirling (1978). Mosquito larvae were reared in tap water at 27°C on a 2:1 mixture of dog biscuit and wheast (yeast grown on milk protein). Larvae of *A. quadrimaculatus* were infected by placing 100 second-instar larvae and 1000 preparasites (infective juveniles) together in 10 ml of deionized water in a petri dish (10 × 35 mm). After 10 min at 24°C, larvae were recovered on a 60-mesh sieve (250-µm pore size), gently rinsed for 2 min with deionized water to remove adhering nematodes, and incubated at 27°C. Larvae were collected for fixation at the following intervals after the 10 min exposure: 5 min, 12 and 24 hr.

TEM methods were adapted from Hayat (1986). Mosquito larvae were collected individually with a pipette, isolated on a 60-mesh sieve, rinsed with deionized water, and transferred to 0.1 M sodium cacodylate (pH 7.4) buffer. Specimens in buffer were added to perforated nylon vials and placed in individual wells of a 24-well tissue culture plate. Five ml of fixative (3% glutaraldehyde in 0.1 M sodium cacodylate buffer) was rapidly added to each vial. After 1 hr at 5°C, larval head and siphon were amputated and the torso transferred to fresh fixative for 2 hr, washed twice in fresh buffer for 1 hr each, and held in the buffer for an additional 12 hr. Fresh buffer was added and specimens were post-fixed for 1 hr in 1% osmium tetroxide. Following osmification, mosquito larvae were washed three times with buffer for 15 min each. Specimens were gradually dehydrated for 25 min each in the following graded acetone series: 30%, 50%, 75%, and 95%. They were then transferred through three changes of 100%

acetone for 3 hr each. Infiltration was performed using a 75:25 starting and 25:75 ending mixture of absolute acetone and Spurr's epoxy. Specimens were transferred to fresh 100% Spurr's epoxy and polymerized at 70°C for 8 hr. Serial sections were cut with glass knives mounted in an ultramicrotome, embedded on formvar-coated 200-mesh grids, and stained with saturated aqueous solution of uranyl acetate for 1 hr and Reynolds lead citrate (Reynolds, 1963) for 5 min. Sections were viewed with a Hitachi H-600 TEM at 75 kv. Hemocytes were located and differential cell counts recorded from 100 total sections through 15 larvae. Hemocytes were identified according to criteria listed by Gupta (1986), hemopoietic tissues after Hoffmann *et al.* (1979), and granules after Rowley and Ratcliffe (1981). Transitional types of hemocytes were not recorded.

RESULTS

Three host hemocyte types were recognized: prohemocytes, granulocytes, and plasmatocytes. Differential hemocyte cell counts revealed prohemocytes were initially the dominant cell type, making up more than half (55%) of all observed blood cells, but their prevalence dropped to less than a quarter (23%) 12 hr later and this cell type was observed infrequently (6%) 24 hr after infection. Conversely, the proportion of granulocytes and plasmatocytes was positively correlated with time post-infection. Granulocytes were initially uncommon (14%), but had increased 2.5 times (35%) in number by 12 hr, and after 24 hr nearly half (46%) of all cell types were granulocytes. Plasmatocytes were the dominant cell type with prevalence increasing from 31% after 5 min to 42% after 12 hrs, and to 48% 24 hr post-infection.

Within 5 min of infection, prohemocytes were found near *R. culicivorax*, often with pseudopodia extended toward the nematode and with vacuoles at the cell periphery nearest the nematode (Fig. 1A, B). Granulocytes observed in contact with nematodes had lysed, releasing granules and other cell products (Fig. 1C). Plasmatocytes were frequently found adhering to nematodes, with pseudopodia spreading over the nematode surface (Fig. 1D, E). Plasmatocytes occasionally lysed to release their cellular contents onto the nematode, resulting in a covering of vacuoles, inclusion bodies, and residual membranes. When present, the nematode surface coat provided a physical barrier between host hemocyte and parasite cuticle (Fig. 1D). When the surface coat was absent, plasmalemma of attached plasmatocytes could be observed interdigitating with the annulations of the nematode cuticle (Fig. 1E1). The surface coat was frequently observed in the process of being shed (Fig. 1F).

By 12 hr post-infection, transitional types of hemocytes were observed. Intact granulocytes were observed in close proximity or in contact with the nematodes (Fig. 2A), and granules in these hemocytes showed unambiguous signs of degeneration (Fig. 2A1). At high magnification, it was apparent that granules were adhering to the *R. culicivorax* surface coat (Fig. 2B). Shedding of the surface coat was also noted (Fig. 2C).

After 24 hr, plasmatocyte remnants could be found on a few nematodes (Fig. 2D). Most granulocytes were in various stages of lysis at or near the parasite, with both mature and immature granules in various stages of degeneration (Fig. 2E). The nematode surface coat was again observed in the process of shedding, with small pieces or flakes of the coat being cast off into the host hemocoel (Fig. 2F).

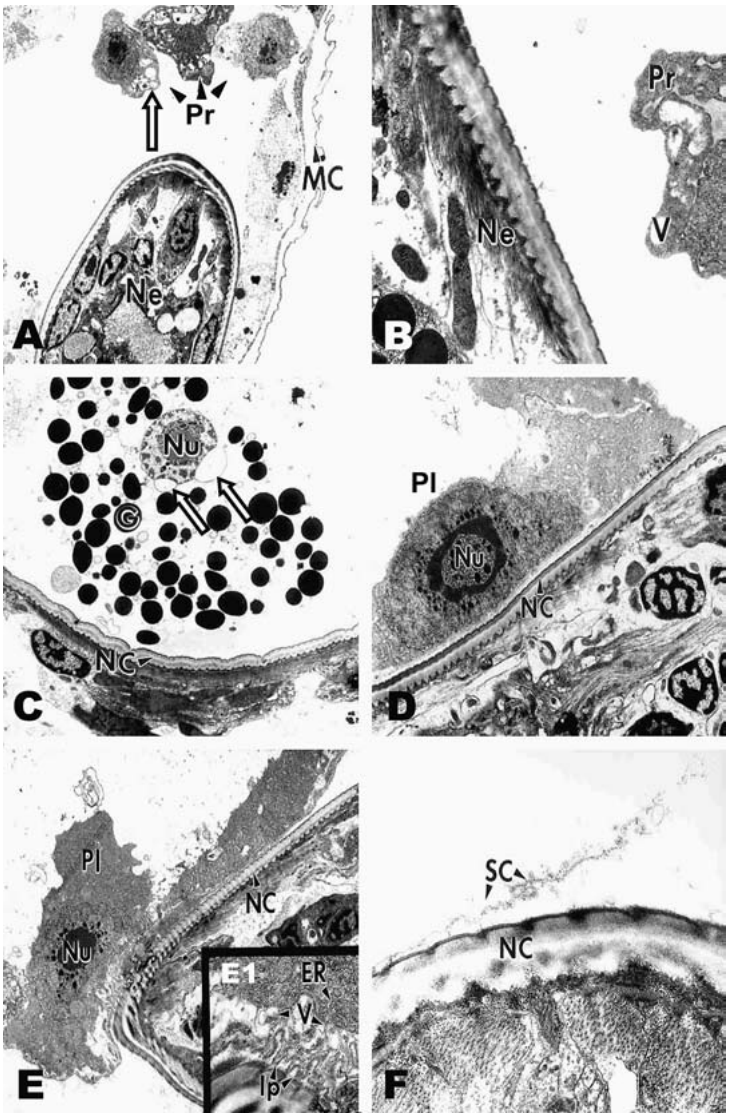

Fig. 1. Transmission electron micrographs of mosquito larvae parasitized by *Romanomermis culicivorax*. A) Prohemocytes (Pr) near a parasitic stage of *R. culicivorax* (Ne) and the abdominal body wall (MC) of an *A. quadrimaculatus* larva at 5 min post-infection. Hemocyte vacuoles are indicated with an open arrow. 4500×. B) A prohemocyte (Pr) from an infected *A. quadrimaculatus* near the nematode *R. culicivorax* (Ne) at 5 min post-infection. Vacuoles (V) are in the cytoplasm nearest to the nematode. No surface coat is detected. 15,000×. C) A lysed granulocyte of *A. quadrimaculatus* with released granules (G) adjacent to the nematode cuticle (NC) at 5 min post-infection. The hemocyte nucleus (Nu) remains intact but shows vacuole formation (open arrows) in the direction of the nematode. 6000×. D) A plasmatocyte (Pl) of *A. quadrimaculatus* spread over *R. culicivorax*; 5 min post-infection. The hemocyte nucleus (Nu) remains intact. The light zone between the hemocyte and the nematode cuticle (NC) is the surface coat. 9000×. E) A plasmatocyte (Pl) with nucleus (Nu) from larva of *A. quadrimaculatus* lysing and spreading over the nematode cuticle (NC); 5 min post-infection. 6000×. Inset (E1) shows hemocyte plasmalemma (lp) interdigitating with the annulations of the nematode cuticle; the endoplasmic reticulum (ER) and a vacuole (V) are also visible. 16,000×. F) Shedding of surface coat (SC) from nematode cuticle (NC); 5 min post-infection in *A. quadrimaculatus*. 30,000×.

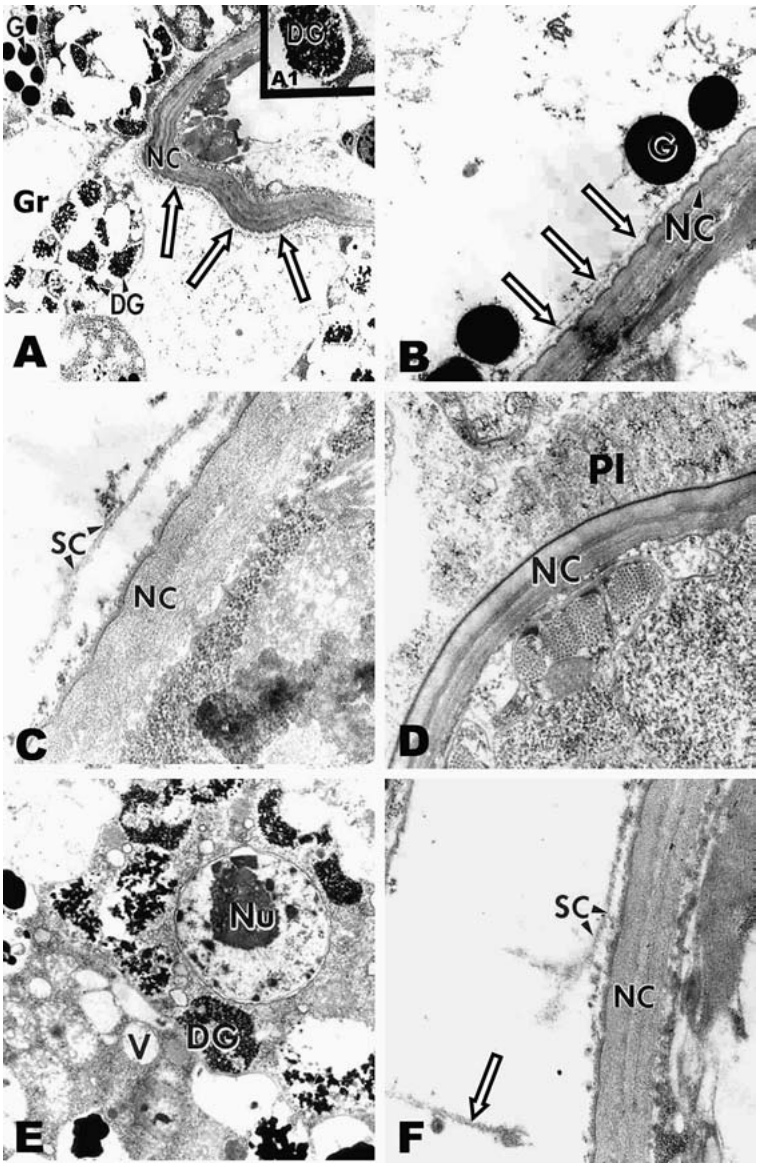

Fig. 2. Transmission electron micrographs of mosquito larvae parasitized by *Romanomermis culicivorax*. A) Granulocytes (Gr) of *A. quadrimaculatus* proximal to the nematode cuticle (NC) at 12 hr post-infection. Many granules (G) are visible; most are undergoing degranulation (DG). The surface coat is visible (open arrows). 6000×. Inset (A1) shows an electron dense granule (DG) during degranulation. 16,000×. B) Discharged granules (G) from a lysed granulocyte of *A. quadrimaculatus* adhering to the parasitic stage of *R. culicivorax* (NC) at 12 hr post-infection. Note the thin surface coat located between the granules and the nematode cuticle (open arrows). 15,000×. C) Shedding of surface coat (SC) from nematode cuticle (NC); 12 hr post-infection in *A. quadrimaculatus* larva. 5250×. D) Remnants of a plasmatocyte (Pl) spread over the cuticle (NC) of *R. culicivorax;* 24 hr post-infection in *A. quadrimaculatus* larva. 18,000×. E) Lysed granulocyte in infected *A. quadrimaculatus* larva; 24 hr post-infection. Intact hemocyte nucleus (Nu), vacuoles (V), and degenerating granules (DG) are clearly visible. 9000×. F) Shedding of surface coat (SC) from nematode cuticle (NC); 24 hr post-infection in *A. quadrimaculatus* larva. Note shed flake (open arrow) of surface coat. 30,000×.

DISCUSSION

Hemocyte recognition of the early parasitic stage of *R. culicivorax* in *A. quadrimaculatus* larvae began immediately after infection. The number of hemocytes in mosquitoes is relatively low (Foley, 1978). We found that as time elapsed, the proportion of plasmatocytes and especially granulocytes increased. These two hemocytes clearly are the key mosquito immunocytes responding to invasion by *R. culicivorax* and their increase indicated an activated host immune system. Degranulation of granulocytes occurred when the host recognized the nematode surface; contact between the hemocyte and the parasite was not required for recognition. Pech and Strand (1996) demonstrated that granulocytes are essential for encapsulation and that neither cell type (granulocytes or plasmatocytes) can form a capsule independently.

Gotz *et al.* (1977) reported melanotic reactions are associated with insect species with low hemocyte counts, resulting in a host response that is humoral and proceeds without direct hemocyte participation. We recorded cellular encapsulation and premelanotic activities in the mosquito-mermithid host-parasite system, a finding in agreement with Poinar *et al.* (1979). Forton *et al.* (1985) concluded that precursors of melanin synthesis are released upon hemocyte lysis in *Ochlerotatus* (=*Aedes*) *trivittatus* Coq. invaded by *Dirofilaria immitis* (Leidy) microfilariae. In the present investigation, we found that discharged and degenerated granules contributed to the formation of acellular electron dense material at the nematode surface. The electron dense material could be the precursor of a melanin layer produced by this host species against nematode invasion (Chen and Laurence, 1985). This mechanism of melanin formation due to granulocyte degranulation is supported by Schmit and Ratcliffe (1977) who reported melanin deposits at the site of granulocyte lysis in larvae of *Galleria mellonella* (L.).

The nematode origin of the *R. culicivorax* surface coat has been established with immunofluorescent techniques (Platzer, 1989). The present study indicates that the sheet-like coat is shed intermittently and serves as a physical barrier between the host immune system and the parasite. The coat often invested the nematode more closely than the loose attachment indicated in a companion SEM study (Shamseldean, unpublished observations) which visualized the nematode exterior, but did not permit the interior cross-section examination perspective afforded by TEM. This suggests the surface coat may initially be tightly attached to the cuticle and is subsequently detached as a pre-shedding step.

The surface cost is an imperfect immunological defense against *A. quadrimaculatus* as the interval between shedding and replacement provided opportunities, albeit limited, for adhesion of hemocytes and their products. This was reflected in observations of hemocyte adhesion and interdigitation with the nematode cuticle when an intervening surface coat was unavailable. Nevertheless, the rate of successful encapsulation is low; less than 3% of invading *R. culicivorax* are inactivated by host hemocyte action (Shamseldean, unpublished data). Because the surface coat is shed in small fragments, only small portions of parasite cuticle are exposed to the host immune system for an unknown duration. Minor accumulations of host immune products are eliminated when the parasite molts after 3 to 4 days. In short, this host is rarely able to exploit the presumably small window of vulnerability the parasite offers.

Further studies of the mermithid surface coat as a counter measure against encapsulation by mosquito immunocytes are warranted. Investigation of the initia-

tion, behaviors, and rates of coat secretion and application to the nematode cuticle are needed, as well as studies on the coat's biophysical properties, composition, and the dynamics of secretion and shedding. These and other studies will permit testing the hypothesis that the surface coat plays a determinate role in mermithid host range breadth.

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