AN ULTRASTRUCTURAL STUDY ON THE EARLY CELLULAR RESPONSE TO *DIROFILARIA IMMITIS* (NEMATODA) IN THE MALPIGHIAN TUBULES OF *AEDES AEGYPTI* (refractory strains)

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Summary :

Dirofilaria immitis living in *Aedes aegypti* refractory strains were studied in relation to ultrastructural events in primary cells of Malpighian tubules and to defense mechanisms activated by host-cells. When the microfilaria reaches the Malpighian cells, its intracellular development is blocked by defense mechanisms activated by the host, resulting in lysis of the outermost cuticle of the parasite without melanin involvement. Ultrastructural evidence suggests that lysis is brought about by Malpighian cell products.

KEY WORDS : *Dirofilaria immitis*, intracellular development, ultrastructure.

MOTS CLES : *Dirofilaria immitis*, développement intracellulaire, ultrastructure.

Résumé : DONNÉES ULTRASTRUCTURALES SUR LA RÉPONSE INTRACELLULAIRE DANS LES TUBES MALPIGHIENS D'*AEDES AEGYPTI* (SOUCHE RÉFRAC-TAIRE) INFECTÉS PAR *DIROFILARIA IMMITIS* (NEMATODA).

Les auteurs décrivent l'évolution de *Dirofilaria immitis* au niveau ultrastructural chez une souche d'*Aedes aegypti* naturellement réfractaire à l'infection. On a observé que les microfilaries gagnent les cellules primaires des tubules malpighiens des moustiques, mais elles ne se développent pas. Après six jours, les microfilaries se trouvent encore dans les cellules primaires des tubules malpighiens, mais elles ne sont pas développées. Dans la plupart des cas, la cuticule des larves montrait des ruptures superficielles et le parasite présentait de nombreux signes d'altérations cellulaires. Toutefois, le processus de mélanisation n'a pas été observé.

INTRODUCTION

Dirofilaria species are nematode parasites that infect domestic and wild mammals. Their development from microfilariae to infective third-stage larvae in mosquito vectors belonging to the genera *Aedes*, *Culex* and *Anopheles* has been the subject of several studies. However, not all species of mosquitoes respond in the same way to these parasites : in species like *Anopheles sinensis* the defense mechanisms of the vector are unable to prevent parasite development (Vegni and Cancrini, 1991), whereas in *Culex* and the *Cellia* subgenus of *Anopheles* the cibarial armature prevents most microfilariae from surviving ingestion and leaving the midgut (Coluzzi and Trabucchi, 1968; Coluzzi *et al.*, 1982; Cancrini *et al.*, 1992). In *Aedes aegypti*, a species without mechanical defense structures, certain strains are refractory to infection with *Dirofilaria immitis* and *D. repens* (Mc Greevy *et al.*, 1974; Coluzzi and Cancrini, 1974). In these strains, Malpighian tubule cells are still invaded by microfilariae but the larvae fail to develop and do not reach the third-stage (Nayar and Sauerma, 1975; Christensen, 1981; Bartlett, 1983; Sauerma and Nayar, 1985; Bradley and Nayar, 1987; Vegni Talluri *et al.*, 1993).

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Little attention has been paid to the mechanisms by which microfilariae are arrested in the Malpighian cells of the host-insect. Bradley *et al.* (1984) described ultrastructural changes in the primary cells of Malpighian tubules of *Ae. taeniorhynchus* following infection with *D. immitis*, with parallel changes in mitochondrial ultrastructure and epithelial transport rates. Bradley and Nayar (1985) demonstrated the presence of melanized larvae of *D. immitis* in primary cells of Malpighian tubules of *Ae. sollicitans*, suggesting humoral melanization at an intracellular site.

In refractory strains of *Ae. aegypti* it has been observed that the intracellular development of *Dirofilaria immitis* microfilaria is blocked by defense mechanisms activated by Malpighian host cells and does not reach the sausage stage (Sauerma and Nayar, 1985). Vegni *et al.* (1993) also found the same situation in *Ae. aegypti* refractory strains infected by *D. repens*.

At the present little is known about what really happens in Malpighian tubule cells invaded by microfilariae or about the first defensive response to the parasite.

The present study was undertaken to examine the ultrastructural events that occur in Malpighian host cells of *Ae. aegypti* refractory strains infected by *D. immitis*, with particular reference to mechanisms which may be responsible for arrest of intracellular development and the death of microfilariae.

Fig. 1. – Scanning electron micrograph of *Dirofilaria immitis*, microfilaria isolated from the lumen of *Aedes aegypti* Malpighian tubule 24 h after infection.

Fig. 2. – *Dirofilaria immitis* microfilaria emerging from primary Malpighian cell. Note the interspace between host-cell and the parasite. Cuticular annulations of the microfilaria are also visible (arrow).

Figs. 3 and 4. – Cephalic and caudal ends of the microfilaria showing annulate cuticle, oral opening (arrow) and anal pore (curved arrow).

Fig. 5. – Electron microscope micrograph of *D. immitis* microfilaria twenty-four hours after the infective blood meal, observed in the cytoplasm of primary Malpighian cells. The nematode (arrows) resides within a clear zone in the cytoplasm. Most of the central cell cytoplasm of the primary cell is occupied by the vacuoles containing electron dense concretions. Small microvilli are also visible (M). Lysosomes (L).

Fig. 6. – Cross section through a microfilaria within a Malpighian cell twenty-four hours after infection. Some vesicles are visible extruding their content into transparent zone surrounding the parasite (arrows). Fig. 7. – Portion of a microfilaria (mf) within Malpighian cell. The cytoplasm of host-cell contains large vacuoles (v) and abundant free ribosomes.

Fig. 8. – Cross section through a microfilaria within a Malpighian cell three days after the blood meal. Note the reduced space between the host-cell and parasite (arrows).

Fig. 9. – High magnification micrograph of microfilaria cuticle three days after infection. Isolated electron dense material is visible adhering to the epicuticle (arrows).

MATERIAL AND METHODS

The refractory strain of *Aedes aegypti* (Liverpool strain) used in this study was selected for refractoriness in 1974 and was originally collected in west Africa.

Three-day-old adult females were allowed to feed on a dog naturally infected by *D. immitis* and were then kept at 26° C and 75 % relative humidity. Twenty mosquitoes, selected at random, were dissected 1, 3 and 6 days after the blood meal. The Malpighian tubules were fixed for 2 h in 5 % glutaraldehyde solution in 0.1 M cacodylate buffer (pH 7.2) and prepared for transmission electron microscopy (TEM). Thin sections mounted on 200 mesh copper grids and stained with uranyl acetate and lead citrate were examined with a Philips CM 10 electron microscope.

Scanning electron microscopy (SEM) observations were conducted on the Malpighian tubules of refractory mosquitoes engorged 24 hrs previously. The larvae were expelled intact by gently crushing the Malpighian tubules under a glass coverslip. The material was fixed, as described above, for 1 hour, repeatedly washed in buffer, dehydrated and arranged on coverslips previously coated with a 1 % aqueous solution of poly-L-lysine. The material was processed by the critical point drying method in a Bomar SPC-900 Ex apparatus. The coverslips with the dried samples were mounted on a base, coated with gold and examined with a Philips KL 20 scanning electron microscope.

RESULTS

Twenty-four hours after the blood meal, SEM observation of the Malpighian tubules of refractory strains of *Ae. aegypti* revealed microfilariae (about 250 µm in length and 3 µm in diameter) intermingled with tubule secretory products (fig. 1). The microfilariae had a corrugated cuticle with transverse striations (fig. 2). The anterior end was bluntly rounded and contained a small mouth opening with a rudimentary buccal orifice surrounded by buccal papillae (fig. 3). The posterior end of the larva showed a whiplike tail with the anal pore opening about 35 µm from the distal extremity (fig. 4).

When thin sections were observed, microfilariae could be recognized inside Malpighian tubule cells, surrounded by an electron transparent area unbounded by a cell membrane (fig. 5).

The cytoplasm of the Malpighian tubule cells had several large vesicles and vacuoles containing concentric electron dense material (fig. 5). Sometimes

the vesicles extruded their contents into the electron transparent material surrounding the parasite (fig. 6). Many free ribosomes and mitochondria and much well developed rough endoplasmic reticulum were also visible in the host-cells (fig. 7).

The intracellular microfilariae (about 5 µm in diameter) did not show ultrastructural abnormalities. The different diameter of microfilariae observed with SEM may be due to differences in fixation methods. The cuticle of the parasite was simple in structure, about 0.22 µm thick and consisted of an epicuticle composed of three dense laminae, a rather uniform fibrillar layer and a relatively thick homogeneous layer separated from the epidermis by a thin dense basal lamella. No cuticle damage was evident (fig. 6).

Three days after the blood meal, cross sections of Malpighian tubule primary cells showed microfilariae (5 µm in diameter) in an essentially identical intracellular position (fig. 8). The cuticle consisted of the same layers as in the 24-hour samples and no signs of degeneration were evident. Many mitochondria were observed in the host-cell cytoplasm near the microfilaria together with smooth and rough endoplasmic reticulum intermingled with vacuoles without of concentric inclusions. An important feature was the reduced space between host-cell and parasite so that the Malpighian cell cytoplasm and the cuticular surface of the parasite were in close contact (fig. 8). Isolated electrondense material was occasionally observed adhering to the epicuticle (fig. 9).

This was also noted in many infected tubule cells of six-day samples. Electrondense material formed a coat where the surface of the larval cuticle adhered to the host-cell cytoplasm (fig. 10). At these points the microfilaria epicuticle appeared to be impaired. The sequence of secretory process leading to the production of the material coating the parasite surface can be summarized as follows : dilated cisterns of rough endoplasmic reticulum (fig. 11), often containing fine fibrous material, and vacuoles accumulate at the edges of the material deposited on the parasite surface; the epicuticle begins to break up where this material is in close contact with the cuticle, and many bubbles appear between the epicuticular laminae of the larva (fig. 12); electrondense material spreads into the basal cuticle layer where the epicuticle is destroyed (fig. 13), invading the underlying larval tissues. No masses of melanin deposits were seen around the larvae.

Cross and longitudinal sections of the microfilariae at various levels revealed damaged internal morphology. Evidence of loss of cell integrity included wide intercellular spaces and degenerative changes such as chromatin clumps close to the nuclear envelope,

Fig. 10. – Six days after the infective blood meal microfilariae (mf) are observed in close contact with the host-cell. Electron dense material, scattered in clusters between cell and parasite surface is visible (arrow).

Fig. 14. – Cross section through a microfilaria within a Malpighian cell six days after infection. Note the signs of degenerative processes of the cells recognizable by the breakage of the contact, cytoplasmic reduction and picnosis of the nuclei.

Figs. 11-13. – Cross sections through a microfilaria within Malpighian cells six days post infection showing the sequences of the electron dense material interposed between the cuticle and host-cell. Fig. 11. Dilated cisterns are visible in proximity of the electron dense material (arrows). Fig. 12. A portion of a microfilaria within host-cell showing the contact between fibrous material and the epicuticle. Bubbles of epicuticular laminae are visible (arrow). Fig. 13. Electron dense material spreading from host-cell cytoplasm inside the microfilaria (mf) at the points where the epicuticle is interrupted (arrows).

degeneration of cytoplasmic components, almost complete disappearance of endoplasmic reticulum, and increase in number of lysosomes and multivesicular bodies (fig. 14).

DISCUSSION

In *Ae. aegypti* and many other mosquitoes the ultrastructural aspects of filarioid larvae destruction concern various melanotic encapsulation responses mainly induced by hemocyte activity. Such melanization processes are reported in species in which the developing site is represented by thoracic muscles (for instance, *Brugia pabangi* and *Wuchereria bancrofti*) as well as in species like *D. immitis* when microfilariae are inoculated intrathoracically into the mosquito. Melanization consists in the deposition of pigment directly on the surface of the parasite by virtue of an interaction with hemocytes in the insect hemocoel; this involves the encapsulation of filarial larvae (Poinar, 1971; Forton *et al.*, 1985; Christensen and Tracy, 1989; Spray and Christensen, 1991).

The defense reactions which occur during microfilarial invasion have also been described in primary cells of Malpighian tubules of *Ae. trivittatus* (Christensen, 1981) and *Ae. sollicitans* (Bradley and Nayar, 1985) infected by *D. immitis*. The authors suggest that the defense reaction consists in a melanotic response of the host-cell to the intracellular parasite, and that the melanin deposits around the cuticle of the larva lead to encapsulation of the parasite. The melanotic response described by these authors and occurring 48-72 hrs after infection, differs somewhat from the defensive response against *D. immitis* microfilariae observed by us in the Malpighian primary cells of the refractory strain of *Ae. aegypti*. Six days after the blood meal, none of infected primary cells showed deposits of dark pigment around the larva, attributable to melanin. At the beginning of infection, electrontransparent material occupied the area between the parasite and the host-cell and a granular coat of electrondense material adhered to part of the cuticle of the parasite. This material, instead of coalescing to form a dense capsule around the larva, adhered to the epicuticle causing lysis of much of the cuticle surface. Through these beats in the epicuticle the dense material came into contact with larval tissues, preventing the microfilariae from developing and migrating into the hemocoel.

The defence mechanism of the primary Malpighian tubule cell can be traced from the moment it is invaded by the parasite. Active penetration of the microfilaria clearly damages the host-cell which has to repair lesions caused by the presence of a very large foreign

organism. If the host-cell is not irreparably damaged, its first step is probably to construct a barrier between its own cytoplasm and the parasite. In fact, 24 h after infection we observed that the space between the host cell and the microfilaria was filled first with homogeneous material released by cytoplasmic vesicles, and then with electrondense granules that coalesced to form clusters of different thickness in various places, in contact with the microfilaria cuticle.

At this stage, three to six days after infection, the contact between the host cell and parasite became more intense. The organelles of the primary Malpighian tubule cells were seen to be in close contact with the interposed electrondense material. The microfilaria began to show signs of degeneration. Where the electrondense material was most abundant, the cuticle was lysed and material produced by the host cell, or other unidentified material entered the larva.

In resistant strains of *Ae. aegypti*, we therefore have a defence mechanism occurring at the first contact of the Malpighian cells with the microfilaria. The process observed by us does not involve melanization and encapsulation of the microfilaria but some kind of defence reaction performed by cells not disposed for immune reactions, like haemocytes, but in any case able to react by isolating the parasite and blocking its development so that it dies. The process by which the cell responds to the parasite may involve intracellular messages from the microfilaria. These may lead to modification of host-cell metabolism in such a way as to activate effector mechanisms that cause lysis of the cuticle and ultimately, destruction of the parasite.

It is evident from the present data and other recent reviews, that the mechanisms by which the intermediate mosquito host is susceptible or otherwise to nematode parasite infection are still unclear. Further immunocytochemical investigation is required to clarify the basis of the defence mechanisms of refractory strains of *Ae. aegypti*.

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