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Defense reactions of *Dermatobia hominis* (Diptera: Cuterebridae) larval hemocytes

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Key Words: *Dermatobia hominis*; hemocytes; phagocytosis; nodule formation; encapsulation

ABSTRACT: The defense reactions against biological (*Histoplasma capsulatum* and *Escherichia coli*) and non-biological materials (China ink and nylon thread) were tested *in vivo* in third instar larvae of *Dermatobia hominis*. The cellular defense performed by larval hemocytes was observed under electron microscopy. China ink particles were phagocytosed by granular cells 5 h after injection. *E. coli* cells were internalized by granular cells as early as 5 min after injection and totally cleared 180 min post-injection, when many hemocytes appeared disintegrated and others in process of recovering. *H. capsulatum* yeasts provoked, 24 h after being injected, the beginning of nodule formation. Nylon thread was encapsulated 24 h after the introduction into the hemocoel. Our results suggest that granular cells were the phagocytic cells and also the responsible for the triggering of nodule and capsule formation. In the presence of yeasts cells and nylon thread, they released their granules that chemotactically attracted the plasmatocytes that on their turn, flattened to surround and isolate the foreign material.

Introduction

Insects are known to possess efficient defense mechanisms against foreign particles. Among these mechanisms are phagocytosis, nodule formation, encapsulation and hemolymph coagulation. Phagocytosis is considered the first barrier against pathogens and it has been described in the hemolymph of many insect species against biological (Ratcliffe and Rowley, 1979; Ratcliffe *et al*., 1985; Götz and Boman, 1985; Ratcliffe, 1986) and non-biological agents (Wiesner, 1991, 1992; Slovák *et al*., 1991). If a considerable number of elements invade the hemocoel, they are isolated by hemocyte aggregation forming nodules melanized or not (Ratcliffe and Rowley, 1979; Lackie, 1980). Encapsulation is performed by the hemocytes when the foreign material is too large to be phagocytosed. Many studies revealed that both humoral and cellular factors contribute to the encapsulation reaction (Götz and Vey, 1987; Götz *et al*., 1987; Rizki and Rizki, 1987). The cellular components (hemocytes) of the immune system of several dipterans have been well studied and five main morphological types were identified: prohemocytes, plasmatocytes, granular cells, adipohemocytes and oenocytoids (Jones, 1962; Whitten, 1964; Rowley and Ratcliffe, 1976; Hall, 1983, Lello *et al*., 1987). Plasmatocytes and granular cells are described as the main cell types involved in all defense mechanisms (Beaulaton and Monpeyssin, 1977; Ratcliffe *et al*., 1985; Ratcliffe and Rowley, 1987; Wiesner and Götz, 1993).

Dermatobia hominis, known as the human bot fly, is an prevalent skin parasite in tropical and subtropical

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America. The larvae of *D. hominis* develop in the subcutaneous tissue of vertebrates, cattle being the preferred host. The parasitic period lasts around 35 days in the summer, when the level of parasitism is higher (Lello *et al*., 1982). Larvae go through three instars, growing from 1 to 25 mm in length and from 0.3 to 10 mm in width, causing the formation of nodules easily noticeable on the skin surface of the host. The third instar larvae weight from 30 to 900 mg, when at the end of this instar they leave the host to pupate. This parasitism is responsible for considerable loss in economy, as it causes decrease in milk production, damage in hide and livestock weight loss. The control of this parasitism has been made by using chemical agents and the impact they may produce in the environment and in the human health is not estimated. To avoid pernicious consequences the biological control would be the solution. The knowledge of the physiology and the defense mechanisms of the parasite is one of the tools to reach this aim.

In this report, we experimentally investigated the role of third larval instar hemocytes of *Dermatobia hominis* in response to different kind of foreign material, such as nylon thread implants, and injections of China ink, *Escherichia coli* and dead yeast *Histoplasma capsulatum* suspensions.

Material and Methods

Animals

Third instar larvae weighting from 400-600 mg were harvest from natural infested cattle by pressing the nodules on the host skin. They were washed in water and separated into four experimental groups.

Injection of test particles and hemolymph collection

Three groups of larvae were injected:

Group 1: Twenty larvae weighting from 400-500 mg were injected into their ventral portion with 2 µl of 0.1%-China ink solution using a hypodermic needle. Half of them were bled after 5 min and half after 5 h post-injection, doing a small cut with a microscissor in the posterior portion of the larvae.

Group 2: Twenty larvae weighting from 500-600 mg were injected into their ventral portion with 2 µl of dead *H. capsulatum* suspension (3.10^7 cells.ml^{-1}) and bled after 24 h as in Group 1.

Group 3: Thirty larvae were injected into their ventral portion with 2 µl of an *E. coli* (Mc Farland.10^{-1}) (ATCC 25922) suspension using a hypodermic needle. They were bled in sub-groups of 10 larvae after 5, 10 or 180 min, as in Group 1.

Hemolymph of each group was dropped into Eppendorf tubes containing Saline Solution for Insects (SSI) (10mM sodium cacodylate; 10 mM $CaCl_2$, 280 mM sucrose, pH 7.4, 380 mOsm.Kg^{-1}) after making a small cut in the larval cuticle with microscissors. Hemolymph was centrifuged twice at 3,000 rev.min^{-1} for 10 min. The pellets were fixed in 2% glutaraldehyde + 2% paraformaldehyde buffered solution and examined by conventional Transmission Electron Microscopy (TEM). Some thin sections of China ink preparations were not post-stained in lead citrate.

Nylon Thread Implants

The implants were carried out by introducing nylon threads into the body cavities of the fourth group of 20 larvae. Ten larvae were dissected after 5 h and the other ten were dissected after 24 h. The recovered threads were fixed in 2.5% glutaraldehyde in phosphate buffer (0.1M, pH 7.3) and post-fixed in osmium tetroxide, dehydrated in ethanol series and substituted by carbon dioxide in a critical point drier. The preparations were mounted in stubs and gold coating was made in a sputter coater and observed in Phillips Scanning Electron Microscope.

Results

Injection of test particles:

China ink solution

After 5 min post-injection, no cellular alterations were observed. Five hours post-injection, although not counted, the number of granular cells in the hemolymph was visibly increased. Pronounced irregularity of granular cells plasma membrane or emissions of pseudopodia were observed, although images suggesting phagocytosis activity by these cells were rarely seen. Small electrondense particles were observed in the extracellular medium and inside of many granular cell vacuoles (Fig.1a). As these observations were inconclusive with the usual preparation for TEM, non-poststained material was observed. It revealed that the vacuole con-

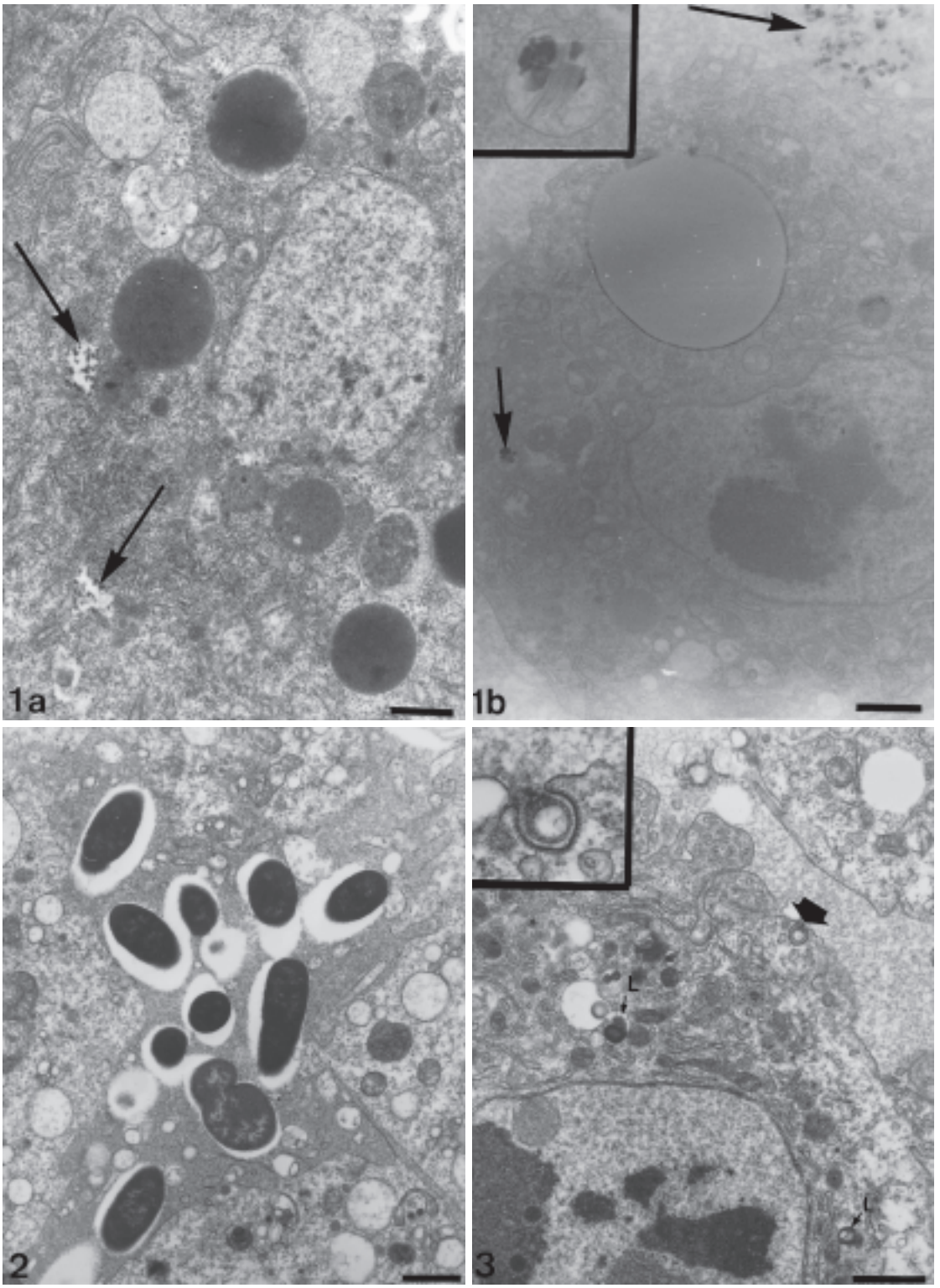


FIGURE 1a-b. Partial view of *D. hominis* granular cell 5 h after China ink injection. **a:** arrows indicate small electrondense particles in vacuoles; bar: 0.5μm. **b:** non-postained cell showing China ink particles in vacuoles and in extracellular portion (arrows); bar: 1μm. **Inset:** high magnification of China ink particle in cytoplasmic vacuole.

FIGURE 2. Dead *H. capsulatum* entrapped by *D. hominis* hemocytes, 24 h post-injection; bar: 1μm.

FIGURE 3. *D. hominis* hemocytes uptaking cell debris (arrow) 24 h after being injected with *H. capsulatum*; L: lisosomes; bar: 1μm. **Inset:** high magnification of coated vesicle.

tents were really China ink particles, as they appeared darker either in the vacuoles or in the extracellular portion (Fig. 1b).

Dead yeasts Histoplasma capsulatum

Within 24 h of injection, an attempt of the hemolymph to isolate the dead yeasts was noticed. Many cells were disrupted and the remainder appeared to be entrapping the yeasts (Fig. 2). Some cells showed remarkable phagocytic activity characteristics, as they appeared uptaking cell debris in coated vesicles. Lysosomes containing ingested debris were seen in their cytoplasm (Fig. 3).

Escherichia coli

Five minutes after injection, noticeable hemocyte alterations were observed. The majority of them exhibited high electrondensity. Some granular cells had large phagosomes containing bacteria (Fig. 4) and some showed signs of degeneration. On the other hand, well-preserved cells were internalizing debris of other disrupted cells. Ten min post-injection, the majority of hemocytes were disintegrated and bacteria were rarely seen among them. After 180 min, no bacteria were found and large amounts of glycogen were laid up in the cytoplasm of the recovering cells (Fig. 5).

Nylon thread implants

Five hours after being implanted, the nylon thread was completely surrounded by fibrin-like, amorphous material plus intact and disrupted granular cells (Fig. 6a,b). Twenty-four hours later, the quantity of fibrous material increased. Besides the intact and disrupted granular cells, spreaded plasmatocytes were clearly seen attached to the clot, characterizing the beginning of the encapsulation of the nylon thread (Fig. 7).

Discussion

Results of this investigation demonstrate that the immune system in *Dermatobia hominis* performs different responses depending on the type, size, and/or number of the foreign material. The period necessary for the hemolymph to perform the defense reaction is another noticeable difference. However, the main hemocytes involved in all the reactions were granular cells and plasmatocytes. Prior studies have shown that these cells are responsible for immune responses in many invertebrates and the fact that they interact to perform the defense mechanisms is not new. In some insects, such as *Galleria mellonella* (Wiesner and Götz, 1993), *Calliphora erythrocephala* (Rowley and Ratcliffe, 1976) and *Euprepocnemis shirakii* (Chang *et al*., 1998), plasmatocytes are considered the phagocytic cells, although granular cells are responsible for this function in *Calpodes ethlius* (Gupta, 1979), *Simulium vittatum* (Cupp *et al*., 1997), and others.

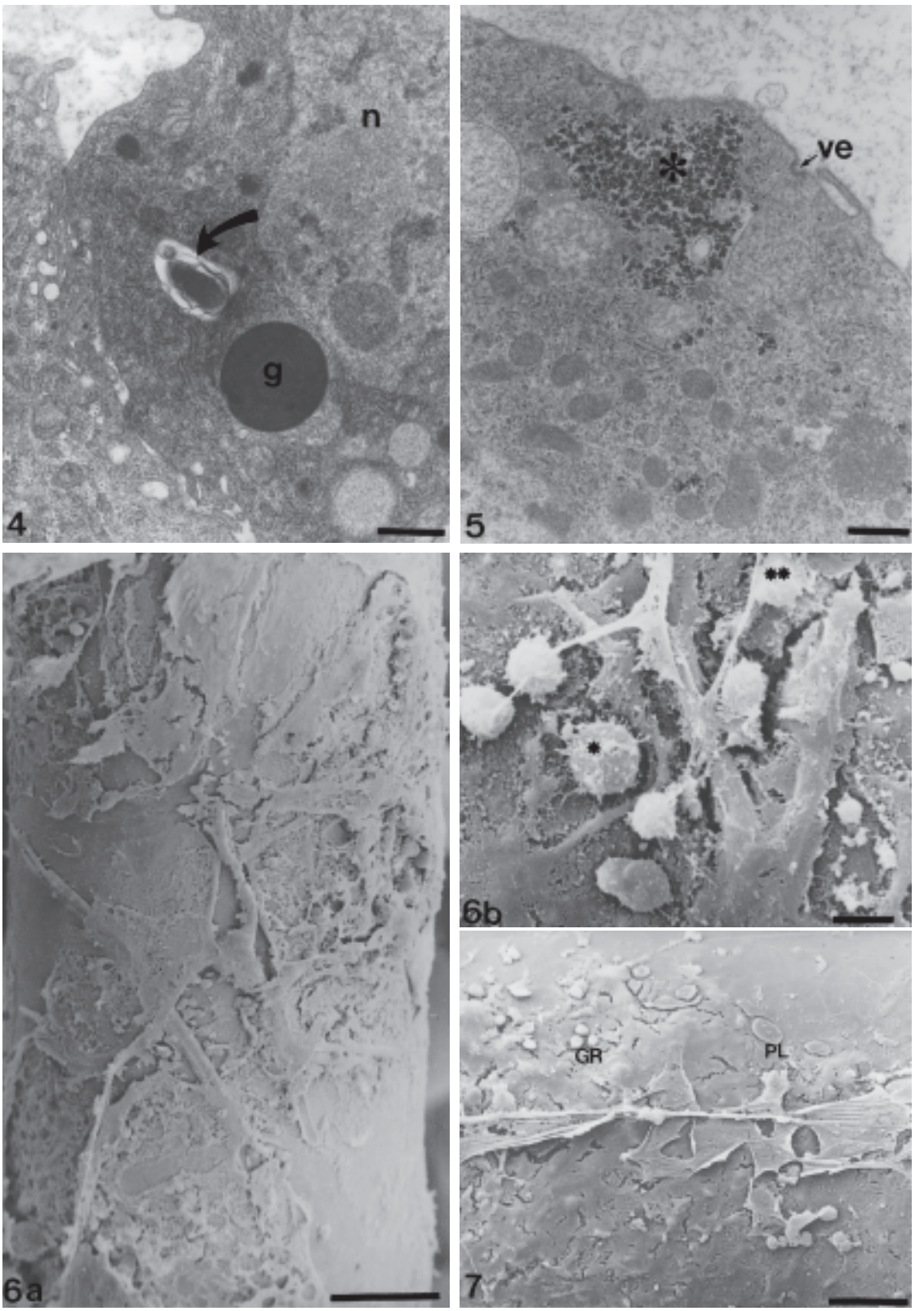
In our results, both China ink and *Escherichia coli* injections provoked phagocytosis by the granular cells. The bacteria were phagocytosed after 5 min and the China ink particles were only seen in granular cell vacuoles after 5 h. Many authors have described phagocytosis of synthetic material by hemocytes of insects (e.g. Neuwirth, 1974; Roe and Kim, 1993; Chang *et al.*, 1998; Cupp *et al*., 1997). Wiesner and Götz (1993) injected *G. mellonella* larvae with hydrophilic and hydrophobic silica beads, each with a well-defined surface structure. They observed that 2.5 h after injection, the granular cells in contact to hydrophilic beads degranulated and this material covered the beads that in sequence were phagocytosed by the plasmatocytes. In contrast, the hydrophobic beads were not attacked by the hemocytes. The mechanisms responsible for the recognition of the synthetic materials can be determined from studies of foreign material in which the surface chemical composition is better defined as different surface characteris-

FIGURE 4. Partial view of *D. hominis* granular cell showing cytoplasmic vacuole containing bacteria (arrow), 5 min after being injected with *E. coli*; g: granule; n: nucleus; bar: 0.5 μm.

FIGURE 5. Partial view of *D. hominis* recovering hemocyte, 180 min after being injected with *E. coli*; (*): glycogen; ve: coated vesicle; bar: 0.5 μm.

FIGURE 6a-b. Partial view of nylon thread, 5 h after being implanted in *D. hominis* hemocoel. **a:** general view showing fibrous material and cells on the surface; bar: 0.1mm. **b:** high magnification showing intact (*) and degranulated (**) granulocytes; bar: 10 μm.

FIGURE 7. Partial view of nylon thread 24 h after being implanted in *D. hominis* hemocoel. Notice granular cells (Gr) and spreaded plasmatocytes (Pl) on the surface; bar: 0.05mm.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tics seem to be responsible for varying capacities of the particles to provoke an immune response. The hydrophilic silica beads used by Wiesner and Götz (1993) provoked, *in vivo*, an intense immune reaction, not described for other synthetic material up to now. The surface characteristics of provocators like China ink particles are not known and therefore there are no valid conclusions about the mechanisms responsible for their recognition (Wiesner, 1992). Our results provide unequivocal evidence that the hemocytes of *D. hominis* are able to phagocytose synthetic particles, even though the particles used here took more time to be recognized by the cells resulting in a delayed phagocytosis.

The mechanisms for recognition of bacteria are better known. Bacterial cell wall components like lipopolysaccharide (LPS) allow a more efficient recognition of these foreign bodies by the hemocytes providing the phagocytosis. Tainai *et al*. (1997) suggest that this cell wall component induces the secretion of cecropin B, an insect antibacterial protein. According to Gillespie *et al.* (1997), these antibacterial proteins and peptides are synthesized by the fat body and hemocytes and then secreted into the hemolymph, where they attack bacteria by several mechanisms. The first step in these mechanisms is the bacterial attachment to the hemocyte surface followed by phagocytosis. Probably, these mechanisms allowed the rapid phagocytosis of *E. coli* by *D. hominis* granular cells.

The picture observed 24 h after *H. capsulatum* injection in *D. hominis* larvae is comparable to the beginning of nodule formation, as many cells were seen disrupted and others entrapping the yeasts. The complete nodule formation was not observed because the larvae were not analyzed after this time. As *D. hominis* larvae are necessarily endoparasites, which raises in a special environment, during our experiments their viability started to decline around 30 h after collected. Da Silva *et al*. (2000) found nodules in *Culex quinquefasciatus* from 24 up to 72 h after injection of *Candida albicans* yeasts. Therefore, probably *in vivo H. capsulatum* could elicit the complete nodule formation in *D. hominis* hemocoel.

Nodules have been studied in insects for decades and their structure and mode of formation are now elucidated (Ratcliffe and Gagen, 1976, 1977; Gupta, 1979; Da Silva *et al*., 2000). Nevertheless, sometimes it is difficult to distinguish nodules from capsules. Most authors agree that the end result of nodule formation is an aggregate of blood cells entrapping the particles in a central melanized region surrounded by a sheath of blood cells (Gupta, 1979). Metalnikov (1924) believes that in nodulation, the association of foreign bodies and hemocytes can occur without prior phagocytosis, and hemocytes form "giant cells" around the particles. In contrast, capsules have flattened, multilayered structures, in which the number of layers can be variable from one species to another (Gupta, 1979). Images observed on the nylon thread surface, 24 h after being in the hemocoel of *D. hominis*, suggest details of the first step in encapsulation. The granular cells release their granules responsible for the chemotactic attraction of the plasmatocytes, which on their turn, spread and form the flattened, multilayered capsule.

The present study emphasizes the importance of the humoral and cellular interaction and cell-cell cooperation in this dipteran species to perform the defense mechanisms. The efficacy of these mechanisms – phagocytosis, nodulation and encapsulation – is the key to understand the huge success of these insects in the nature.

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