

MACROPHAGE-NEMATODE INTERACTION *IN VIVO* :
Nippostrongylus brasiliensis
INFECTIVE LARVAE IN THE PERITONEUM
OF UNSENSITIZED RATS¹

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SUMMARY. Infective larvae of *Nippostrongylus brasiliensis* injected into the peritoneum of non-immune rats become coated with several layers of host macrophages. Cell-coated larvae remain free in the peritoneum or attach to the omentum, while other larvae attach to the omentum without becoming coated. All larvae, whether coated or not, die, become pigmented and break into fragments. Ultrastructural observations revealed a progressive disintegration of the soft tissues of the immobilized larvae, characterized by autolytic changes and accumulation of pigment with histochemical characteristics of lipofuscin. The cuticle remains intact and excludes Trypan blue during the entire autolytic process, thus demonstrating that macrophage secretions do not participate in the disintegration process. When larval enzymes are inactivated by heat treatment and the larvae are injected i.p., no autolysis occurs and the internal organs remain recognizable. These larvae become coated over their entire length with several layers of macrophages and are progressively phagocytized by macrophages clustering at both ends. Different modes of nematode disintegration within the rodent host involving macrophages are discussed.

Key-words : *Nippostrongylus brasiliensis* infective larvae. Peritoneal cavity. Macrophage adherence. Autolysis. Larval enzyme inactivation. Phagocytosis. Lipofuscine.

Interaction macrophage-nematode *in vivo* : larves infestantes de *Nippostrongylus brasiliensis* dans le péritoine de rats non sensibilisés

RÉSUMÉ. Les larves infestantes de *Nippostrongylus brasiliensis*, inoculées dans la cavité péritonéale de rats non immuns, sont rapidement couvertes par plusieurs couches de macrophages. Ces larves restent libres dans la cavité péritonéale ou s'attachent à l'omentum ; des larves dépourvues du manteau de macrophages peuvent également s'attacher à l'omentum. Toutes les larves, recouvertes ou non de macrophages, meurent : elles se pigmentent et se brisent en fragments.

Les observations ultrastructurales de ces larves montrent une désintégration progressive des tissus mous, caractérisée par des changements autolytiques et par l'accumulation de pigment

1. This work was supported in part by a grant to the first author from the Kuvim Center for the Study of Infectious and Tropical Diseases, The Hebrew University-Hadassah Medical School, Jerusalem, Israel.

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Accepté le 13 mai 1986.

ayant les caractéristiques histochimiques de la lipofuscine. La cuticule reste intacte pendant l'autolyse (les larves ne se colorent pas au bleu trypan). Ceci démontre que les sécrétions des macrophages ne participent pas à cette désintégration.

Quand des larves, dont l'activité enzymatique a été détruite par la chaleur, sont inoculées par voie intrapéritonéale, il n'y a pas d'autolyse. Les organes internes restent intacts. Ces larves, comme les larves injectées vivantes, sont aussi rapidement couvertes de plusieurs couches de macrophages. Elles sont progressivement phagocytées par les macrophages, qui s'accumulent aux deux extrémités des larves. Nous discutons les divers modes de désintégration des Nématodes chez le Rongeur dans les cas où interviennent les macrophages.

Mots-clés : *Nippostrongylus brasiliensis*. Larves infestantes. Cavité péritonéale. Attachement des macrophages. Autolyse. Inactivation des enzymes larvaires. Phagocytose. Lipofuscine.

Introduction

Larvae and adult nematodes have been shown to provoke a cellular response *in vivo* resulting in damaged and destroyed parasites. This process has been observed with several nematode species in normal and immune hosts.

Parasites introduced into an immune host are damaged by the active participation of the host macrophages. In mice, immunized to *Nippostrongylus brasiliensis* and exposed to a percutaneous challenge, the larvae become immobilized in the dermis and are surrounded by macrophages and fibrocytes. Worm destruction begins with dissolution of the cuticle followed by breakdown of the tissues (Lee, 1977).

Among several specimens of *Dipetalonema setariosum* recovered from a long standing infection of a jird (presumably immune), one was found bearing a small cell mass made up exclusively of macrophages which appear to secrete an electron dense material causing damage to the cuticle. Macrophages were also seen inside the nematode, thus attacking the cuticle "from both the inner and outer aspects" (Worms and McLaren, 1982).

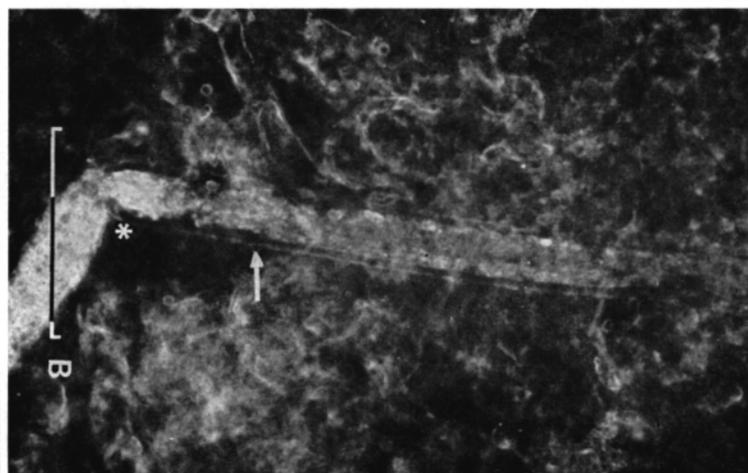
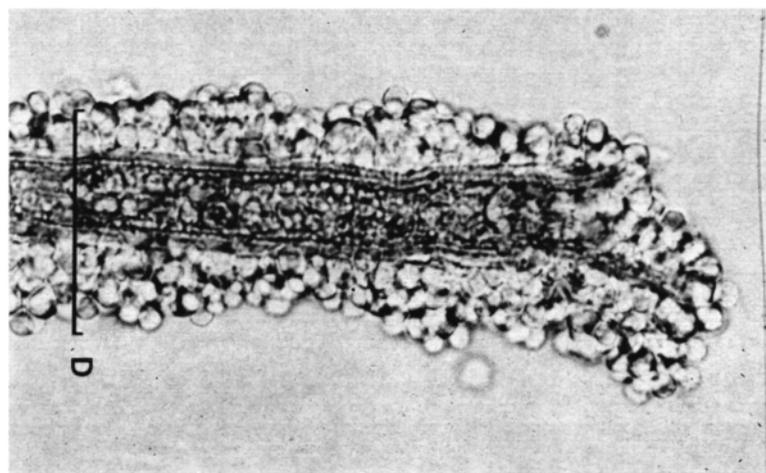
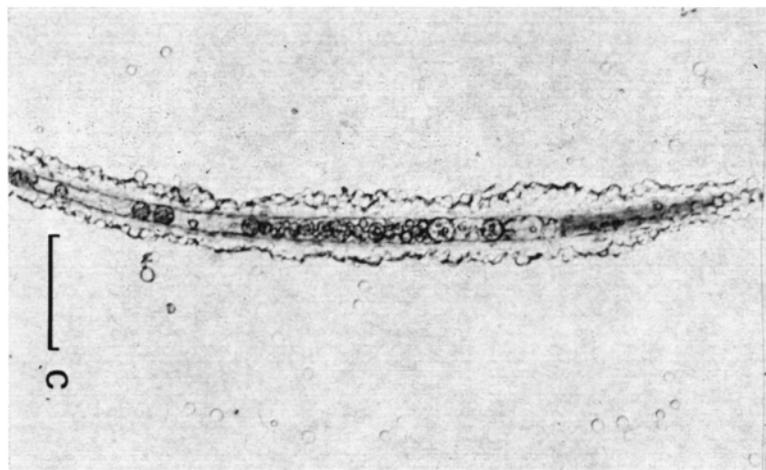
Larval disintegration within the normal host also involves host macrophages, but their role appears to differ from that in the immune host. When *N. brasiliensis* infective larvae were injected intraperitoneally (i.p.) into normal mice, the worms were recovered from the peritoneum covered with (adhering) host cells (Crandall, Crandall and Areat, 1967). In long term experiments with *N. brasiliensis* infective

FIG. 1. — A) Pigmented fragments of a *Nippostrongylus brasiliensis* larva within a granuloma recovered 9 days p.i. with living larvae. Bar line represents 100 μ m.

B) Larva injected alive recovered 4 days p.i. Cuticle has remained intact (arrow) although tissue is becoming pigmented and beginning to fragment. Bar line represents 100 μ m.

C) Enzyme-inactivated larva recovered 5 days p.i. Larva coated with macrophages, internal organs recognizable, Trypan blue staining. Bar line represents 50 μ m.

D) Enzyme inactivated larva recovered 4 days p.i. Mummified larva with posterior end partly phagocytosed, Trypan blue staining. Bar line represents 50 μ m.



larvae injected i.p. into normal rats, the macrophage coated larvae which remain free in the peritoneal cavity or adhere to the omentum, become organised into granulomas in which the larvae die, fragment and darken (Greenberg and Wertheim, 1973; Wertheim and Hamada, 1980).

The present study was undertaken to analyse the macrophage-parasite interaction and mechanisms of larval destruction on the level of the light and electron microscope using living and enzyme inactivated (heat killed) *N. brasiliensis* infective larvae introduced i.p. into naive rats.

Materials and Methods

Male rats of the outbred Hebrew University Sabra strain, 150-180 g, were used in these studies. Infective larvae were isolated from 10 day old fecal cultures, washed and surface sterilized as previously described (Greenberg and Wertheim, 1973). For heat killing (enzyme inactivation) at 80 °C the larvae were introduced into a preheated test-tube and covered rapidly with several ml of heated physiological saline.

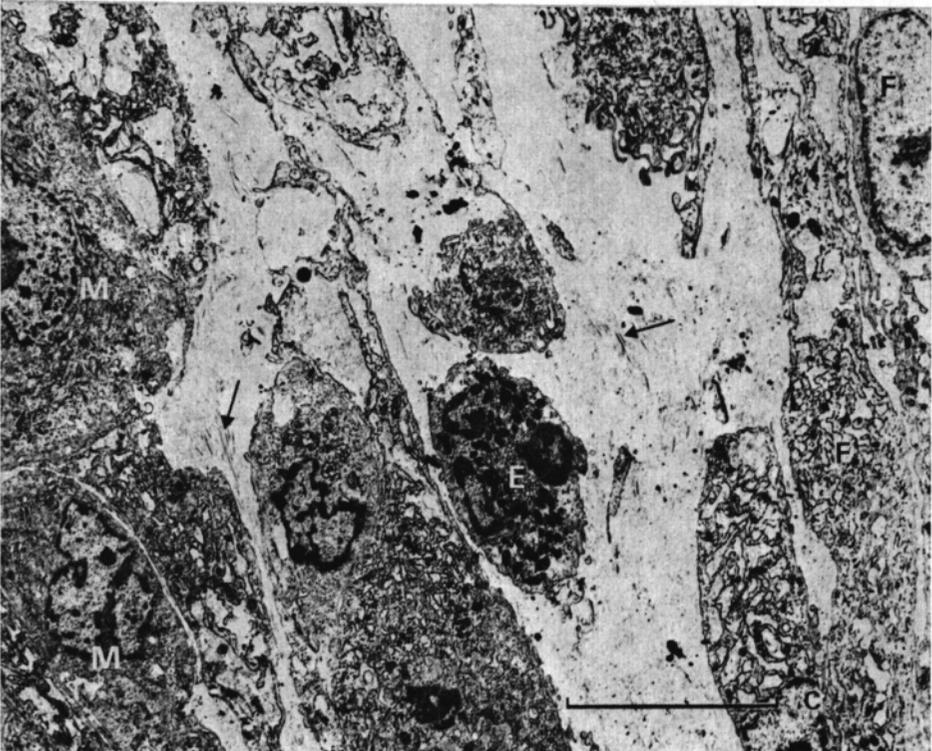
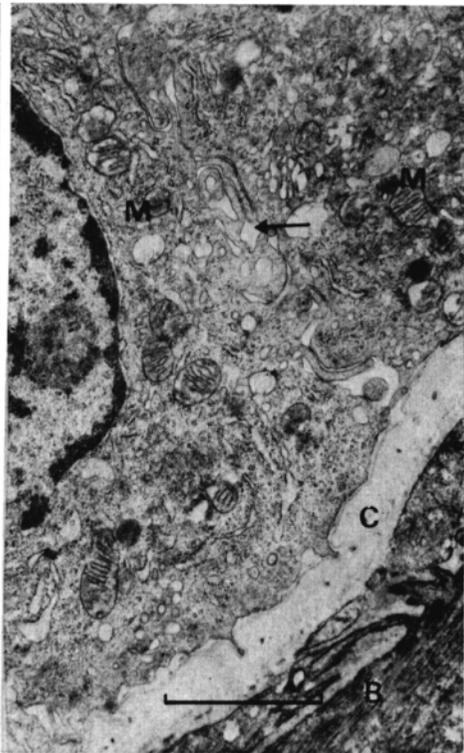
The inoculum consisted of 4,000 larvae in 0.5 to 1.0 ml saline injected i.p. in the lower left quadrant of the abdomen. The animals were killed with ether and examined at 15 min, 6 h and 1, 2, 3, 6, 7 days post infection (p.i.). To recover the parasites, 6 ml of phosphate buffered saline (PBS) were injected into the peritoneum, the abdomen lightly massaged, washed with alcohol and incised at mid-line. The exudate containing coated larvae was aspirated with a Pasteur-pipette, and transferred to saline. Single larvae were placed into drops of a 2.5 % (w/v) solution of trypan blue to check cuticle permeability. The omentum was spread on a wet glass plate and cut into pieces containing larvae; the preparation was then examined after staining with trypan blue. For electron microscopy, free larvae and those attached to the omentum were cut with a scalpel to facilitate penetration of the fixatives. Tissues were fixed for 2 h in cold 2.5 % (v/v) glutaraldehyde in 0.1 M cacodylate buffer pH 7.4 and, after washing in the same buffer, postfixed for 1 h in cold cacodylate-buffered 1 % (w/v) OSO_4 . Nematodes were embedded in epon by standard procedures and sectioned on a LKB Ultratome. Sections were stained

FIG. 2. — A) *Nippostrongylus brasiliensis* third stage larva recovered 24 h after i.p. injection. Detail of initial contact between microvilli of host macrophage and larval cuticle. Bar line represents 1 μm .

B) Third stage larva recovered 7 days after i.p. injection. The host macrophages adhere closely to the larva with microvilli projecting into the annular grooves of the cuticle. The lateral microvilli of the adjacent macrophage interdigitate (arrows) to form the initial enclosing layer of host cells. Bar line represents 2 μm .

C) Cross section of the granuloma formed by host peritoneal cells around a larva recovered 6 days after i.p. injection. Macrophages form inner layer adjacent to larva. Fibroblasts form the outer layer of granuloma and deposits of collagen fibers are present (arrows). An eosinophil is also present. Bar line represents 10 μm .

M : Macrophage — C : Cuticle — E : Eosinophil — F : Fibroblast.



with uranyl acetate followed by lead citrate, and examined with a Philips 300 electron microscope at 60 KV.

Histochemical procedures were used to characterize the yellowish-brown pigment granules deposited in moribund larvae. Larvae adhering to pieces of omentum were fixed in neutral buffered formalin, embedded in paraffin and sectioned at 8 μm . Entire larvae and sections were bleached in 10 % (v/v) hydrogen peroxide or in peracetic acid (Pearse, 1972). Sections were treated with Nile blue sulphate to differentiate lipofuscin from melanin. Prussian blue was applied to sections pretreated with 10 % hydrogen-peroxide as a test for free and masked iron.

Results

Light Microscopic Observations

Living larvae injected i.p. became covered with macrophages over their entire surface, as previously described (Greenberg and Wertheim, 1973). Within the cellular coat the larvae displayed tissue disintegration and fragmentation associated with an accumulation of yellow-brown pigment. The cuticle remained visibly intact and no peritoneal cells were observed within the cuticle. Eventually, granulomas were observed surrounding short, deeply pigmented, cylindrical fragments (*fig. 1 A*), resulting from cuticle breaks at the point of tissue fragmentation (*fig. 1 B*).

Coated larvae remaining free in the peritoneum survived and were motile longer than those attached to the omentum. Many uncoated larvae that attach to the omentum became filled with pigment and fragmented like the cell-coated ones. Living larvae from the inoculum, as well as larvae in all stages of disintegration, excluded trypan blue. Trypan blue entered the broken ends of larval fragments but did not penetrate the cuticle covering these fragments.

Following i.p. injection, enzyme-inactivated larvae became coated with macrophages like those injected alive. However, no tissue disintegration, fragmentation, or pigmentation was observed (*fig. 1 C*). In these cell-coated larvae recovered 6 days p.i. the internal organs could be recognized. Larvae recovered at a later date had

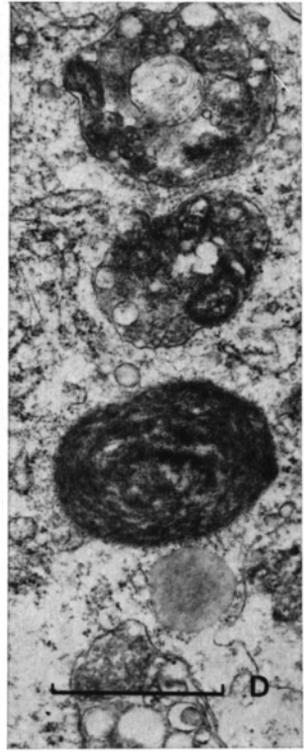
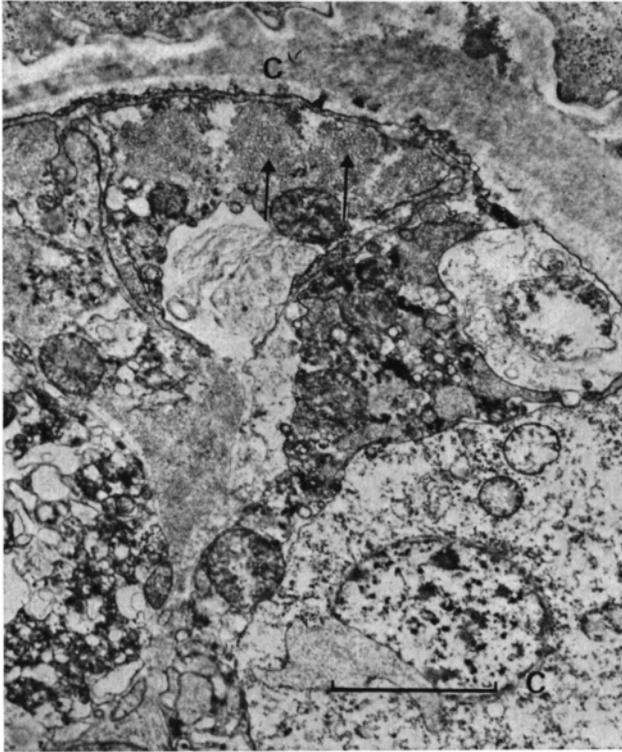
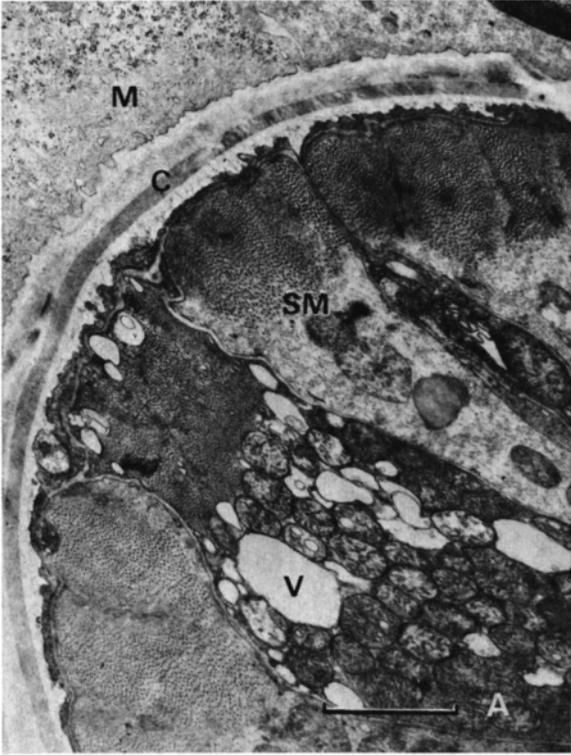
FIG. 3. — A) *Nippostrongylus brasiliensis* third stage larva recovered 6 h after intraperitoneal injection. Host macrophages adhering to larva. Cuticle intact and larval somatic muscle cells easily identifiable. Cytolysis has begun with mitochondrial disruption and formation of vacuoles. Bar line represents 2 μm .

B) Detail of mitochondria from *N. brasiliensis* larva recovered 6 h after i.p. injection. The mitochondria are distended, and the membrane and matrix are disrupted. Bar line represents 0.5 μm .

C) Third stage larva recovered 24 h after i.p. injection. Cuticle is intact, there is extensive cellular disruption, the myofibrils (arrows) are affected but still recognizable. Other cells are not identifiable. Bar line represents 2 μm .

D) Third stage larva recovered 24 h after i.p. injection. Detail of autolysosomes from a degenerating larval cell. Bar line represents 1 μm .

M : Macrophage — C : Cuticle — SM : Somatic muscle — V : Vacuole.



the anterior and/or posterior ends missing and macrophages were observed clustered at the damaged ends (*fig. 1 D*). Phagocytosis from both ends continued causing the larvae to become shorter and shorter until they completely disappeared. No macrophages were seen within the larval tissues. The larval cuticle is permeable to trypan blue 6 h p.i.

The nature of the deeply pigmented tissue fragments observed in larvae injected alive was examined histochemically. The pigment granules in sections were bleached for 24 h with 10 % H_2O_2 or with peracetic acid. When stained with Nile blue sulfate and decolorized with 10 % H_2O_2 the granules remained blue. In sections pretreated with peroxide and stained with Prussian blue, the granule proved not to contain iron. These staining characteristics indicated that the pigment was lipofuscin and not melanin.

Electron Microscopic Observations

MACROPHAGES

The macrophage was the predominant cell type in direct contact with the larval cuticle. Villous projections of the macrophage made initial contact with the cuticle (*fig. 2 A*) after which the cell firmly attached itself to the worm. The interdigitated lateral villous projections of adjacent macrophages contributed to the formation of a compact layer around the larva. Deposits were not observed between the macrophage membrane and larval cuticle (*fig. 2 B*). Lymphocytes, eosinophil and neutrophil granulocytes as well as additional macrophages adhered to the innermost layer of macrophages closely adhering to the cuticle and the cluster became a granuloma, bounded by fibroblasts and deposits of collagen fibers (*fig. 2 C*).

Most of the adhering macrophages remained unaltered during the adhesion process even when the larval tissues became completely lysed and replaced by pigment deposits. In macrophages, apparently close to fractures in the cuticle, pigment was seen in the spaces between surrounding cells and in the cells, causing alterations in their organization (*fig. 4 D*).

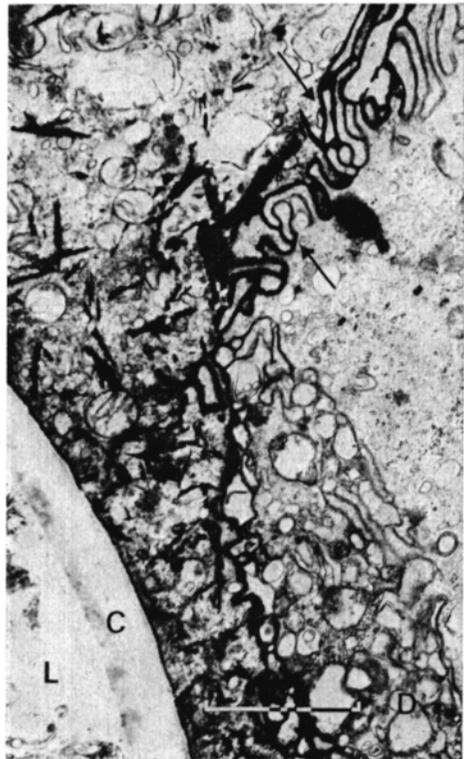
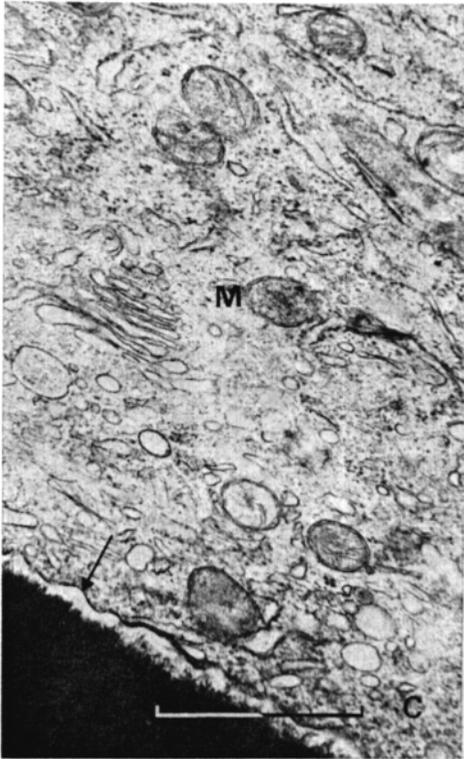
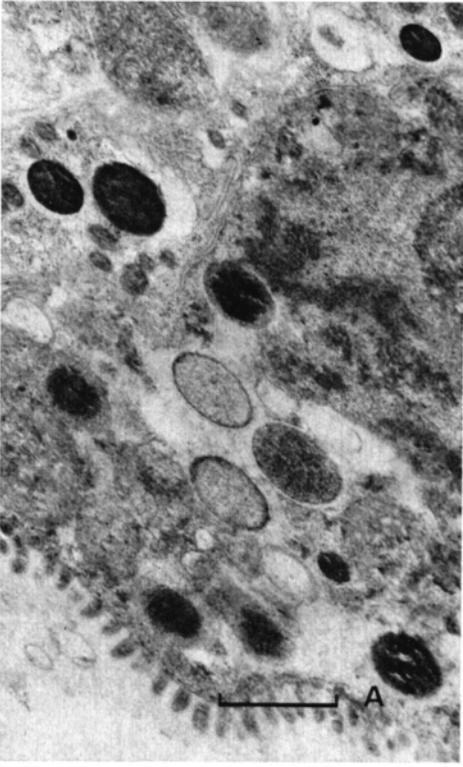
FIG. 4. — A) *Nippostrongylus brasiliensis* third stage larva recovered 72 h after i.p. injection. Intestinal cell showing pigment in oval membrane bounded granules. Granules contain varying amount of pigment. Bar line represents 1 μ m.

B) Third stage larva recovered 72 h after i.p. injection. Hypodermis containing pigment in the form of small to large, irregular dense granules. Bar line represents 1 μ m.

C) Portion of granuloma formed around a larva recovered 12 days after i.p. injection. The entire larva is filled with pigment with only the outer cortex of the cuticle still intact (arrow). The adhering macrophage appears normal. Bar line represents 1 μ m.

D) Portion of granuloma formed around a larva recovered 6 days after i.p. injection. Larval tissues lysed, cuticle intact, striated layer still visible. Pigment in the space between contiguous macrophages (arrows) and necrosis of the innermost cells. Note needle-like pigment aggregates in innermost cells. Bar line represents 1 μ m.

H : Hypodermis — M : Macrophage — C : Cuticle — L : Larva.



THE PARASITE

The process of larval disintegration and pigmentation was studied with the EM in larvae that had been injected i.p. alive. Since the process was not synchronous, the time after injection at which a given change was first observed is indicated in the protocol.

The first morphological signs of the larval disintegration was vacuolization and distension of the mitochondria, as well as vacuolization and membrane disruption in some muscle cells. The cuticle remained intact in the observed areas and the muscle fibers unaltered (*fig. 3 A and B*). A more advanced stage of disruption was indicated by large empty spaces, tissue necrosis and numerous autolysosomes (*fig. 3 C and D*). After three days the deposits of pigment were seen in the hypodermis and in the cells of the intestine. Pigment in the intestinal cells appeared to accumulate in oval, membrane bounded granules, whereas in the hypodermis the pigment deposits were irregular in outline and appeared to coalesce into large aggregates (*fig. 4 A and B*). Eventually the entire larva became filled with pigment with only the outer cortex of the cuticle remaining intact. No pores or holes in the cuticle were observed in the electron micrographs studied (*fig. 4 C and D*).

Discussion

Macrophages are implicated in destruction of immobilized or weakened parasites in immune hosts by dissolution of the cuticle and larval tissues (Lee, 1977 ; Worms and McLaren, 1982). In the present study, destruction, death and fragmentation is observed in *N. brasiliensis* larvae enclosed in a macrophage coat with the cuticle unimpaired. The cuticle excludes trypan blue and the outer cortex remains intact even in the late stages of larval pigmentation and fragmentation (except at the fracture points). While Worms and McLaren (1982) observe layers of dense material, construed to be lytic secretion, between the cuticle and the adhering macrophage, no such layer was seen in the present study. Thus, larval destruction is not caused by macrophage secretory activity.

Ultrastructure evidence in this study demonstrates that larval destruction is a result of autolysis of the larval tissue. Tissue disintegration proceeds by cell vacuolization, formation of autolysosomes and the accumulation of pigment. This conclusion is strengthened by the fact that when the worms are exposed to a temperature sufficient to disrupt enzymatic activity, larval autolysis does not occur. In these enzyme-inactivated larvae, recovered as late as 6 days p.i., the internal organs are recognizable and pigmentation does not occur. However, heating alters the cuticle, making it trypan blue permeable, and the macrophages are now able to phagocytose the larva beginning at the anterior and posterior ends.

Since the disintegration of larvae injected alive is not caused by the enzymatic activity of the adhering cells but by an autolytic process, the question arises as to how this autolysis is initiated. The fact that uncoated larvae trapped by the omentum

undergo autolysis, pigmentation and fragmentation just like the coated ones suggests that the autolytic process is triggered by larval immobilization. This is further strengthened by the observation that in a larva partly adhering to the omentum, only the immobilized portion pigments while the rest of the larva continues to move for some time.

Histochemical tests identified the pigment in the disintegrating larvae as lipofuscin. Inclusions of lipofuscin were recently demonstrated in intestinal cells of ageing *Caenorhabditis briggsae* but not in young worms. Pigment deposition was therefore considered a process correlated with ageing and a process of accumulation of waste products (Epstein, Himmelhoch and Gershon, 1972), similar to that occurring in mammalian cells (Fawcett, 1966). The finding of lipofuscin in the present study implies that this pigment may form not only in ageing cells but also during autolysis in nematode larvae.

Three different types of macrophage activities appear to operate *in vivo*, damaging or destroying nematode parasites. In the immune host, immobilized or weakened worms are attacked by macrophages that actively damage the cuticle and gain access to the inside of the worm (Lee, 1977; Worms and McLaren, 1982). Second, in naive hosts *N. brasiliensis* larvae with the cuticle impaired by heating become covered with adhering macrophages, which do not enter into the worm body but phagocytize it from the ends. Third, in naive hosts unimpaired larvae become immobilized by adhering layers of macrophages and are destroyed by autolysis.

REFERENCES

- CRANDALL C. A., CRANDALL R. B., AREAN V. M. : Increased resistance in mice to larval *Ascaris suum* infection induced by *Nippostrongylus brasiliensis*. *J. Parasitol.*, 1967, *53*, 214-215.
- EPSTEIN J., HIMMELHOCH S., GERSHON D. : Studies on ageing in nematodes. III. Electron-microscopical studies on age-associated cellular damage. *Mech. Ageing Dev.*, 1972, *1*, 245-55.
- FAWCETT D. V. : The Cell, its Organelles and Inclusions. *W. B. Saunders Co.* Philadelphia and London, 1966.
- GREENBERG Z., WERTHEIM G. : The cellular responses of the rat to an intraperitoneal inoculation of *Nippostrongylus brasiliensis* larvae. *Immunology*, 1973, *24*, 531-43.
- LEE D. L. : Ultrastructural changes in the infective larvae of *Nippostrongylus brasiliensis* in the skin of immune mice. *In* : Studies in Parasitology in Memory of Clark P. Read (J. E. Byram, L. Stewart, Eds). *Rice University Studies*, 1977, *62*, 175-182.
- PEARSE A. G. E. : Histochemistry, Theoretical and Applied. Vol. II, 3rd. Ed. *Churchill Livingstone*. Edinburgh and London, 1972.
- WERTHEIM G., HAMADA G. : *Nippostrongylus brasiliensis* : Quantitative and qualitative aspects of larval destruction in the peritoneum of the rat, *Isr. J. Med. Sci.*, 1980, *16*, 560. (Abstract).
- WORMS M. J., MCLAREN D. J. : Macrophage-mediated damage to filarial worms (*Dipetalonema setariosum*) in vivo. *J. Helminthol.*, 1982, *56*, 235-241.