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Ultrastructural Changes in *Enterobius vermicularis* from Man Treated with Ma-Klua and Combantrine

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Introduction

Diospyros mollis is a species of woody tree belonging to the Ebenacea family. It grows wild in the northern and middle parts of Thailand and bears yellowish-green fruit during the rainy season. Ma-Klua, the antihelmintic berries of *D. mollis,* has been used as an effective drug for intestinal parasites by the native herb doctors in Thailand since old times. The antihelmintic effects of juice extracted from this fruit have been demonstrated by Sadun and Vajrasthira (1954), Mokkhasmit and Pengsritong (1967), Sen *et al.* (1974) and Srinophakun *et al.* (1978). According to Mokkhasmit and Pengsritong (1967), excellent anthelmintic efficacy against intestinal parasites was obtained in almost all cases in man, although many of the patients required more than one treatment. The chemical nature of Ma-Klua consisting of diospyrol was elucidated by Borsub *et al.* (1976), Nilanidhi and Prachankadee (1962), Yoshihira *et al.* (1971) and Tezuka *et al.* (1973).

Morphological research has been done briefly on a few helminths after Ma-Klua treatment by Koyama *et al.* (1980) with a light microscope and Tongu *et al.* (1980) with an electron microscope. To date, however, there has been no detailed account of the ultrastructural changes on the worm organs such as cuticle, muscle and intestine. The present study describes the morphological changes in *Enterobius vermicularis* caused by Ma-Klua and Combantrine by electron microscopy.

Materials and Methods

The Ma-Klua powder used for this experiment was extracted from fruit of *Diospyros mollis* by alcohol, and was kept under vacuum in hermetically sealed ampules. One dose of 50 mg/Kg of Ma-Klua was administered to each patient infected with *Enterobius vermicularis* in Thailand in January, 1978. Another vermicide, Combantrine (10 mg/Kg), was given to other patients. Adult worms of *E. vermicularis*

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were collected from the feces of the patients. The worms were immediately fixed in 4% phosphate-buffered glutaraldehyde solution (0.1 M, pH 7.4) at room temperature and were maintained in the same fixative solution for 2 weeks at ambient temperature in Thailand and in Japan until they were processed for ultrastructural studies. Then the specimens were cut into several pieces, each 2 or 3 mm long. After 3 or 4 rinses in the same buffer, the specimens were fixed in 1% phosphate-buffered osmium tetroxide solution for 2 hours or more, dehydrated in ethanol by a routine method, and embedded in Epon mixture after passing through n-butyl glycidil ether. Thin sections were cut with a Porter-Blum ultramicrotome, double stained with uranyl acetate and lead citrate, then observed with a Hitachi HS-8 electron microscope at an accelerating voltage of 50 kV. Normal worms were collected from the perianal region of a child in Japan for control when they migrated naturally from the anus. After being fixed with glutaraldehyde solution, the worms were examined in much the same way as in the case of Ma-Klua and Combantrine treatment.

Results

(1) Morphological features of control worms

The cuticle of *Enterobius vermicularis* was divided into 6 major layers of different electron density in longitudinal section: 1, external-cortical layer; 2, middle-cortical layer; 3, internal-cortical layer; 4, homogeneous layer; 5, inner-homogeneous layer; 6, basal layer. The hypodermis (Fig. 1, H) was situated beneath the cuticle (Fig. 1, C) and was composed of a thin layer of syncytial cells between the cuticle and muscle layers. The hypodermis had many mitochondria, vacuoles of various sizes, and collagenous fibers. The muscle cell (Fig. 1, MC) consisted of a contractile and a noncontractile portion and was located beneath the hypodermis. The contractile portion included thick and thin myofilaments, the noncontractile portion mitochondria, nucleus, and other organelles.

The intestinal wall consisted of a single layer of intestinal cells (Fig. 2, I) having a brush border of microvilli (Fig. 2, V) facing on the intestinal lumen. These intestinal cells contained a nucleus, mitochondria, and many vacuoles. The intestine was surrounded by a homogeneous basal lamina (Fig. 2, BL). Furthermore the intestine was sparsely covered by a thin muscle layer around the basal lamina.

The egg shell of *E. vermicularis* consisted of 2 layers, the outermost slit layer (Fig. 3, SL) and an inner chitinous layer (Fig. 3, CL). The slit layer showed many lattice structures at right angles to the shell surface and small canals radiating from inside to outside in several places. The inner chitinous layer exhibited homogeneous less-dense structures.

(2) Morphological changes caused by Combantrine

The cuticle (Fig. 6, C) which was treated with Combantrine showed structures similar to the cuticle of control worms. In the hypodermis (Fig. 6, H), the vacuolation (Fig. 6, V) became gradually clear. The mitochondria in the muscle cell (Fig. 6, MC) gave indications of swelling in noncontractile portions. The microvilli (Fig. 5, V) of intestinal cells (Fig. 5, I) was much the same structure as the control. However, the matrix of nucleus (Fig. 5, N) contained some dense bodies and vacuoles. Endoplasmic reticulum and many glycogen-like granules were increased in the cytoplasm of the intestinal cells (Fig. 5, I).

(3) Morphological changes caused by Ma-Klua

Marked changes occured in the worms treated with Ma-Klua. The 6 layered cuticle (Fig. 8) lost its distinct stratum. The outermost layer, the external-cortical layer

(Fig. 8, EL), became more dense than those of Combantrine treated or control worms. Moreover many dense granules (Figs. 7, 8, 11) composed of 2 kinds of shape were deposited in the cuticle. The round-shaped granules were mostly located in the middle-cortical layer (Fig. 8, ML) beneath the external-cortical layer (Fig. 8, EL). On the other hand, the needle-shaped ones were distributed in a mass in the cuticle, the hypodermis (Figs. 7, 8, H), the muscle cell (Fig. 7, MC), and especially the muscle nucleus (Fig. 12, N). However, both granules were not found in the intestinal cell (Figs. 9, 10, I).

The needle-shaped granules were gathered in groups in the internal-cortical layer under the transverse striations in the longitudinal section (Fig. 7, TS). The intestinal cell showed collapse accompanying the swell of mitochondria and an increase in intercellular space. Therefore, the microvilli (Fig. 10, V) on the lumen surface were seen indistinctly. Some bacteriae (Figs. 9, 10, B) appeared in the intestinal lumen and even in the intercellular space between intestinal cells. The outermost half of the slit layer (Fig. 4, SL) of the egg shell in the uterus became more electron-dense than that of control or Combantrine treated worms. No changes were seen in the embryo.

Discussion

Antihelmintic efficiency of Ma-Klua against various intestinal parasites, especially nematods, has been demonstrated in man and experimental animals by several workers (Mokkhasmit and Pengsritong, 1967; Sen *et al.*, 1974; Srinophakun *et al.*, 1978; Sadun and Vajrasthira, 1954). However, the morphological changes of various organs of *Necator americanus* and *Enterobius vermicularis* after treatment with Ma-Klua were referred to as only two brief notes by Koyama *et al.* (1980) and Tongu *et al.* (1980). According to light microscopy by Koyama *et al.* (1980), dark brown granules of Ma-Klua were seen in the cuticle, hypodermis, intestinal cell, and genital organs of *N. americanus* and *E. vermicularis*, and these granules were particularly aggregated in the hypodermis and intestinal cell which might be high in the metabolism. In the present observations with the electron microscope, the aggregation of granules was distributed throughout the cuticle, hypodermis, and muscle cell as in the case of light microscopy by Koyama *et al.* (1980). However, the granules were absent from the intestinal cell. Furthermore, 2 kinds of granules consisting of round-shaped and needle-shaped granules were identified in the organs of *E. vermicularis*. In the cuticle, the needle-shaped ones were located in most layers except for the external-cortical layer, and the round-shaped ones were mostly in the middle-cortical layer of the 6 layers that were classified by Tsubota (1966). The strongest correlation was suggested between these granules and Ma-Klua, because the granule was absent in the control and Combantrine treated worms. It dose not seem that there is fundamental difference between 2 types of granules. Ma-Klua seems to show different shapes when it is recrystallized in the worm tissues. The fact that the granules of Ma-Klua were gathered in groups beneath the transverse striations suggests the uptake of Ma-Klua through these portions which are very thin. The nucleus of muscle cells was packed with many needle-shaped granules. It seems that the nucleus has an affinity for Ma-Klua. According to Comley (1980), major changes in the intestinal lumen and cell of *Aspiculuris tetraptera* after *in vivo* treatment with mebendazole and thiabendazole by electron microscopy included a marked reduction in the microbial flora (bacteriae) and the appearance of large numbers of autophagic vesicles in the cytoplasm of the intestinal cells. In the present study on *E. vermicularis,* the intestinal cell which was treated with Ma-Klua suffered great damage, i.e., collapse of the intestinal cell and villi. However, granules as in the cuticle, hypodermis and muscle cells were not fined in the intestinal cell. Nevertheless it should not be concluded that Ma-Klua was not absorbed from the intestine even though the granules were absent from intestinal cell. The intestinal cell which was damaged indicates the possibility of absorption of Ma-Klua by this cell. Ma-Klua may exist as solution in the intestinal cell. Many bacteriae were seen in the intestinal lumen in comparison with control worms. Ma-Klua seems to have no effect upon these bacteriae. Inatomi (1957) divided the egg shell ultrastructure of *E. vermicularis,* into 2 layers, compact-outer (slit layer) and inner-looser (chitinous layer). In the present observations the egg shell surface of the slit layer in the uterus was dyed densely by Ma-Klua. The embryo, however, remained much the same structure as in the case of control or Combantrine treated worms. This drug probably could not pass through the egg shell.

As concerns worms treated with Combantrine, the organs such as cuticle, hypodermis, muscle cell, and egg showed very similar morphology to that of control worms. The intestinal cell, however, showed signs of degeneration; namely, the matrix of nucleus contained dense bodies and vacuoles, and cytoplasmic reticulum and glycogen particles in the cytoplasm were increased in the number. The precipitation granules seen in the case of Ma-Klua were not identified anywhere. This indicates that the reaction mechanisms in *E. vermicularis* are different for Ma-Klua and Combantrine.

Summary

Enterobius vermicularis treated with the anthelmintics, Ma-Klua and Combantrine, in man in Thailand was examined morphologically by electron microscopy. In the worm after Ma-Klua treatment, the outermost layer of the cuticle (outer-cortical layer) and egg shell surface (slit layer) became electron-dense. Many electron-dense granules consisting of 2 types, round and needle-shaped granules, were distributed in most organs such as cuticle, hypodermis, and muscle cell except the intestinal cell. These granules were gathered in groups especially in the inner-cortical layer under the transverse striations of the cuticle. This is suggestive of absorption of Ma-Klua from the cuticular surface, particularly from the transverse striations. The granules were also packed into the muscle nucleus which might have a specific affinity for Ma-Klua. The intestinal cells showed collapse of microvilli and cytoplasmic organelles. This collapse is probably the result of the reaction of the intestinal cell to Ma-Klua. The worms treated with Ma-Klua were different from those treated with Combantrine in terms of the morphology of the cuticle, hypodermis, muscle cell, egg shell, and intestinal cell.

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Ma-Klua 及び Combantrine の蟯虫に及ぼす微細形態学的影響

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昔からタイ国で腸管内寄生虫の駆除のため使用されて来たマクルアの蟯虫体に及ぼす微細形態学的影響を観察した．使用した薬剤は *Diospyros mollis* の果実よりアルコール抽出，乾燥粉末として真空アンプルに保存したものを用いた．これの 50 mg/kg とコンバントリンの 10 mg/kg をそれぞれ別のタイ国の蟯虫患者に投与し，濾便で得られた虫体を直ちに 4 %のグルタールアルデヒドで前固定した．その後この液のまま日本に持帰り通常の後固定を経て電子顕微鏡で観察した．マクルアで駆虫したものは角皮，角皮下層，筋細胞内に電子密度の高い二種類の顆粒の沈着がみられた．特に角皮では輪状線の直下に集合が認められ，角皮より吸収されることが示唆される．筋細胞では特に核内に多数の針状顆粒が集積され，マクルアと核との親和性が高いと考えられる．腸管壁細胞は崩壊が進んでいるにもかかわらず前記のような顆粒は認められない．この事実はマクルアが微絨毛より吸収されないと考えるよりも腸壁の細胞内では代謝機能等の相違により水溶性の形で存在するものと考えられる．コンバントリン駆虫体ではマクルアに於るような顆粒の沈着は認められず，コントロールに較べてほとんど形態的な差は認められない．ただ腸管細胞だけは核内に空胞，顆粒が出現し，細胞質内にも多数の空胞化が起って細胞の退化像が認められる．この事実はコンバントリンが腸管微絨毛より吸収されていると考えられる．

Explanation of Figures

Fig. 1 Cuticle(C), hypodermis(H) and muscle cells(MC) of control *Enterobius vermicularis*.

Fig. 2 Intestinal wall of control worm consisted of intestinal cell(I) having many vacuoles, basal lamina(BL) and muscle layer. IL: intestinal lumen, V: microvilli.

Fig. 3 Egg in the uterus of control worm. The egg shell consists of two layers, slit layer(SL) and chitinous layer(CL).

Fig. 4 Section of the egg in the uterus treated with Ma-Klua. The outer surface of the slit layer(SL) became more dense than control. CL:chitinous layer.

Fig. 5 Section of the intestinal wall treated with Combantrine. The intestinal cell(I) contains a nucleus(N) with dense bodies and vacuoles. V:microvilli, IL:intestinal lumen.

Fig. 6 Longitudinal section of the body wall treated with Combantrine. The muscle cells(MC) have many swollen mitochondria(M). C:cuticle, H:hypodermis, V:vacuole.

Fig. 7 Longitudinal section of the body wall treated with Ma-Klua, showing aggregate granules under the transverse striations(TS). C:cuticle, H:hypodermis, MC:muscle cells, M: mitochondria.

Fig. 8 Longitudinal section of the cuticle treated with Ma-Klua, showing six layered cuticle. EL:external-cortical layer, ML:middle-cortical layer, IL:internal-cortical layer, HL: homogeneous layer, IH:inner-homogeneous layer, BL:basal layer, H:hypodermis.

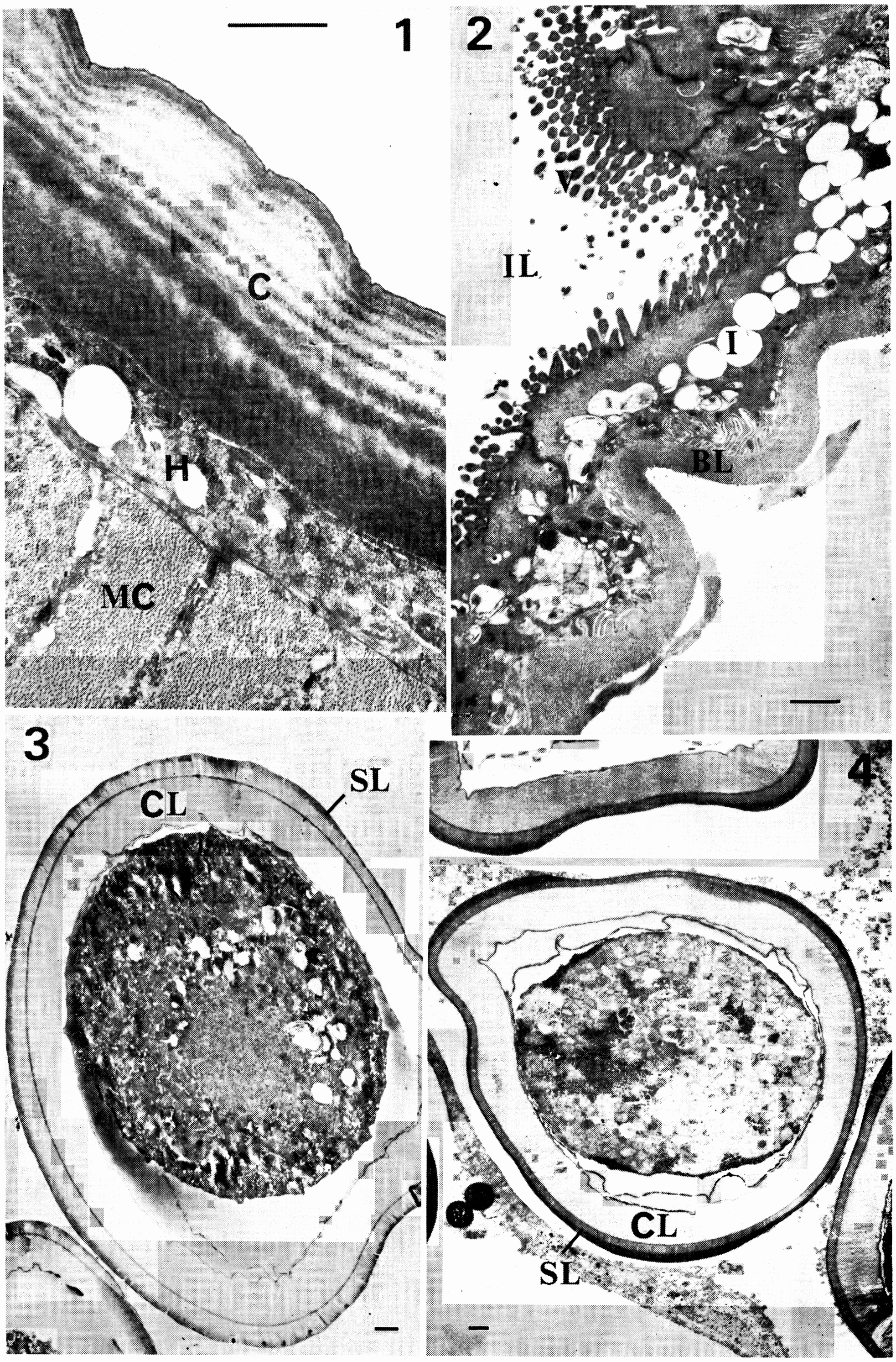
Fig. 9 Showing the collapse of intestinal cell (I) treated with Ma-Klua. The gap between intestinal cells (arrow), and intestinal lumen containes many bacteriae (B). BL:basal lamina.

Fig. 10 Higher magnification of the same worm as Fig. 9. The intestinal cell has unclear microvilli(V). B:bacteria, IL:intestinal lumen, I:intestinal cell.

Fig. 11 Showing 2 typed granules in the cuticle treated with Ma-Klua. NG:needle-shaped granule, RG:round-shaped granule.

Fig. 12 A muscle nucleus (N) treated with Ma-Klua is packed by many needle-shaped granules. There are many swollen mitochondria(M) in the cytoplasm. MC:muscle cell.

(Bar is one micron in each figure)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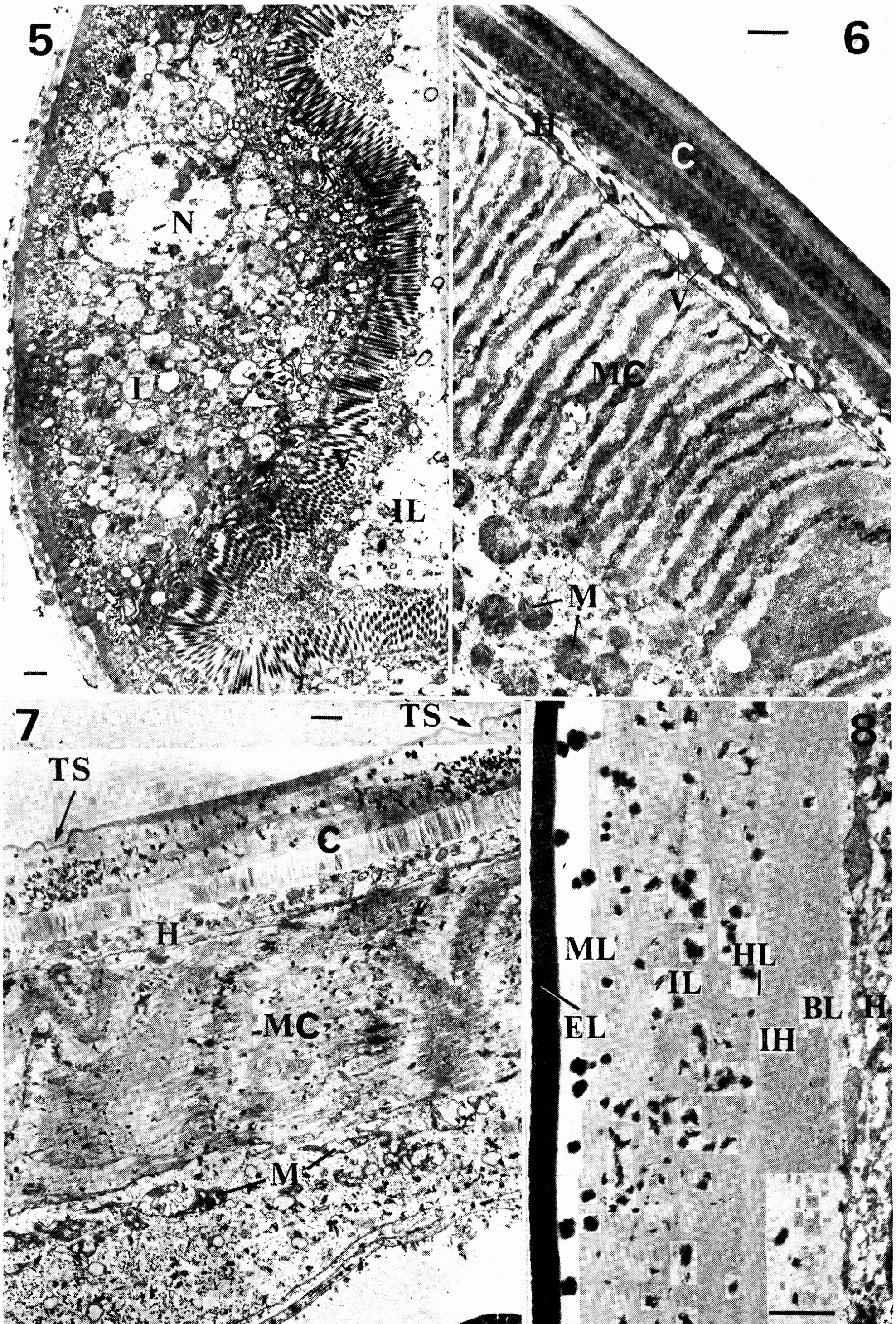
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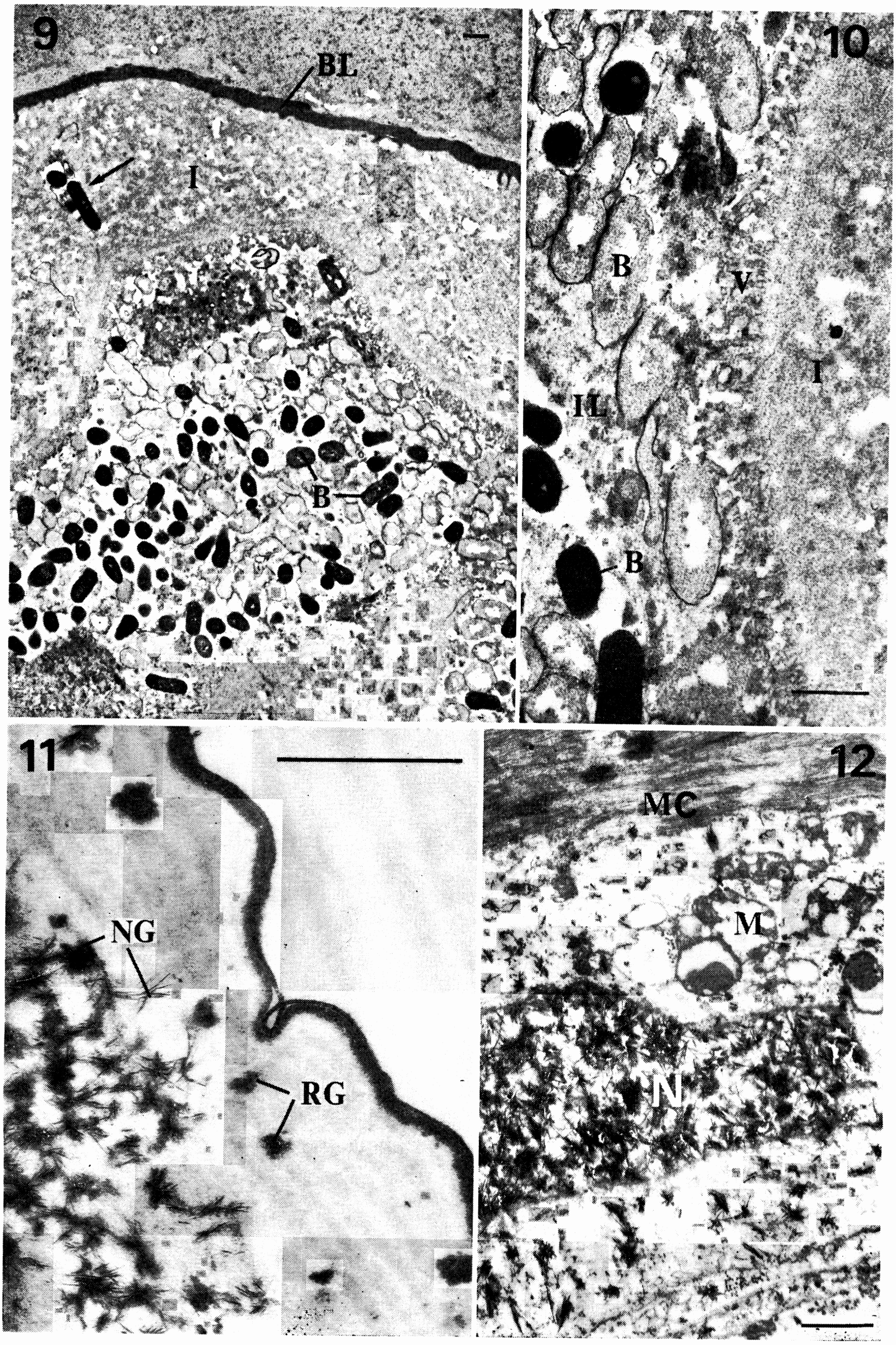
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