Ultrastructure of the Cuticle of *Brugia pahangi*: a Rich Source of Antigen

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Abstract

The cuticle is a rich source of antigen of the filarial parasite, *Brugia pahangi*, as revealed by indirect immunofluorescent study. Transmission electron microscopy reveals that the cuticle of the L_3 and adult *B. pahangi* is surrounded by a discontinuous trilaminate membrane-like epicuticle. This is one of the richest sources of antigens, because this structure is in direct contact with host immunity. The cuticle comprises the basal, middle and cortical layers, with no clear demarcation between each layer. All layers comprise fine filamentous structures arranged in several directions. These fibrils of the cuticle are believed to be collagen-like protein, but comprised of finer fibrils, with no periodicity. These cuticular proteins may be slowly turned over and released to the environment and act as one source of immunogen. The hypodermis shows cellular components in the lateral cords. Each cell bears organelle characteristics of highly synthetic activity and infolded plasma membranes at both apical and basal regions. Therefore, in addition to its role in synthesizing cuticular proteins, the infolded plasma membrane may play roles in controlling and facilitating the exchange of nutrient and waste materials through the cuticle. These excreted materials may also be another source of antigen.

Keywords: ultrastructure, cuticle, *Brugia pahangi*

Introduction

Brugia pahangi, an animal parasite, is closely related to human lymphatic filariae, and it has been illustrated to have antigenic homology with human *Brugia* species [1]. Meizels *et al* [2] studied the cross-reactive surface antigens on three stages of *B. malayi*, *B. pahangi* and *B. timori* by radio-iodination and immuno-precipitation. They found that the surface antigens had a characteristic pattern in each stage, and the adults and L_3 had relatively more complex patterns than microfilariae. Furthermore, the surface antigens of the three stages of these three species were all closely homologous. Immunoprecipitation revealed that antibody raised in mice against one stage or species reacted with surface antigens from other stages and species. They also showed cross-reaction with stage-specific antisera, which suggested that there must be shared epitopes on *Brugia* surface antigens from each stage. Moreover, Meizels [3] showed that the surface antigens of adult *B. pahangi* were also recognized by antibody from patients with *Wuchereria bancrofti*, *Loa loa* and *Mansonella perstans*. Our immunofluorescent study clearly confirmed commonness among

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antigens from various *B. malayi, B. pahangi* and *W. bancrofti* tissue sources. At the molecular level, only a few defined antigens of selected interest, such as the major adult surface protein of MW 29,000 appeared to be recognized in all stages of the parasite [4]. Another dominant cross-reacting antigenic determinant of these filarial species is phosphorylcholine, as noted by Gualzata *et al* [5]. This haptenic group was present in many different components, including a proteoglycan-like polymer found in the circulation of *Onchocerca, Wuchereria* and *Brugia*- infected people [6-8]. This hapten was so immunodominant amongst filarial antigens that it was difficult to resist the conclusion that antiphosphorylcholine antibody could be protective to the host [9-12].

Because of shared antigenicity among *B. pahangi* and human filarial species, it seems possible that the antigens released by these parasites may be mostly similar and originate from the same tissue source. Therefore, research with *B. pahangi* on antigens of immunodiagnostic potential may be readily applied to other human filarial species. If this is the case, work to define useful antigen immunodiagnosis, and to develop vaccine, could be greatly simplified, since *B. pahangi* can be kept in cycle more easily than human filariae. The ultrastructure of *B. pahangi* should also be carefully studied to elicit more detail of all antigenic structures. Further work at the molecular level is obviously needed to identify the antigens from various tissue sources before any realistic application can be contemplated.

Materials and methods

The adult worms were recovered from the peritoneal cavities of Mongolian jirds (*Meriones unguiculatus*), which had been previously infected by injecting infective stage larvae into the peritoneal cavities 60 days earlier, by the method of McCall *et al* [13]. The jirds were sacrificed, the abdominal cavities were exposed by making a small incision line, the adult worms were collected by two pairs of tweezers, and then washed several times in PBS.

The third stage larvae were obtained by dissecting *Aedes aegypti* (Liverpool strain) mosquitoes that had been fed on an infected cat 12 days previously.

B. pahangi parasites were obtained from London School of Tropical Medicine and Hygeine, United Kingdom. Specific diagnostic points of *B. pahangi* microfilariae and adults are as follows:

microfilaria: innerkorper length = 53.1 (44-63) mm, acid phosphatase staining, acid phosphatase activity along entire body;

adult female: absence of minute cuticular bosses at the tail region;

adult male: sharply pointed tip of spicule.

For ultrastructural studies of the adult and L_3 of *B. pahangi* by transmission electron microscopy (TEM), the parasite specimens were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 at 4˚C for two hours. They were then washed three times with the same buffer, post-fixed in 1% osmium tetroxide (OsO_4) in 0.1 M sodium cacodylate buffer, pH 7.2 at 4˚C for one hour, and washed three times with distilled water. After tertiary fixation and staining with 1% uranyl acetate in distilled water for 20 minutes, the parasites were washed three times with distilled water. They were dehydrated in graded series of ethanol (50-100%) for 15 minutes at each step and then infiltrated twice in propylene oxide for 20 minutes each. The solution was replaced twice with mixtures of propylene oxide and absolute alcohol at 1:2 and 2:1, respectively, and left in the 2:1 mixture overnight; they were then embedded in Araldite. Thin sections showing silver to gold interference were mounted on formvar-coated copper grids and further stained with uranyl acetate and lead citrate for 30 minutes each, and observed by transmission electron microscope.

Results

Cuticle of adult *B. pahangi*

The cuticle of the adult worm is approximately 2-2.5 mm thick but varies slightly among different regions and levels of the worm body. The thickest part is opposite the lateral cord. The cuticle comprises the basal, middle and cortical layers with no clear demarcation between each layer, a feature similar to many other nematodes (Figs 1, 2). The cortical layer is subdivided into the external and the internal layers. The surface covering of the external cortical

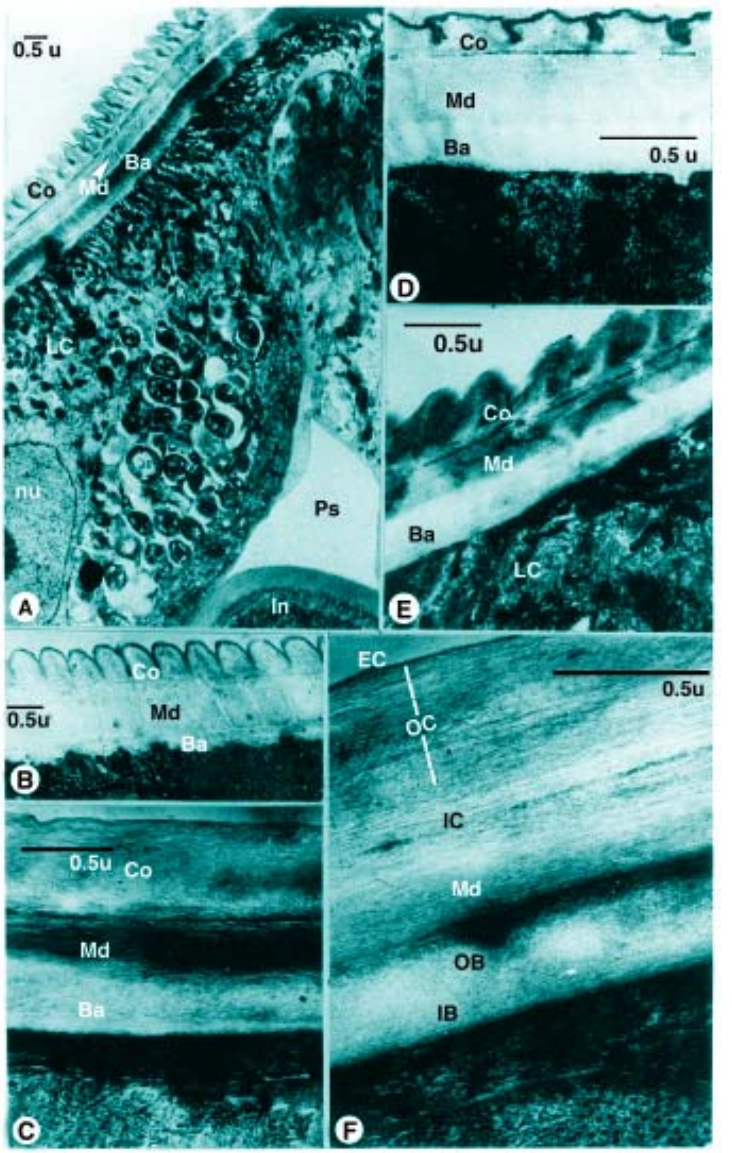

Fig 1 Transmission electron micrographs (TEM) of adult *B. pahangi* cuticle: A) The lateral cord (LC) with euchromatic nucleus (nu) and somatic musculature (M) are covered by the scallop-shaped cuticle. These are components of the body wall covering the body cavity or pseudocele (Ps), which is occupied by the gut (In = intestine) and reproductive tract. B-E) Higher magnification of the cuticle showing different shapes of annulations and the subdivision of layers of the cuticle. The cuticle can be divided into three layers: cortical (Co), middle (Md) and basal (Ba). F) Higher magnification showing the fibrillar components of the cuticle, this revealing the different directions of the fine fibrillar structure. The cortical layer is covered externally by the epicuticle (EC), an osmiophillic trilaminate membrane-like structure. Owing to the different directions of the fibrils, the cortical layer is further divided into two sublayers: the outer cortical (OC) and inner cortical (IC); the basal layer is also divided into another two sublayers: the outer basal (OB) and inner basal (IB).

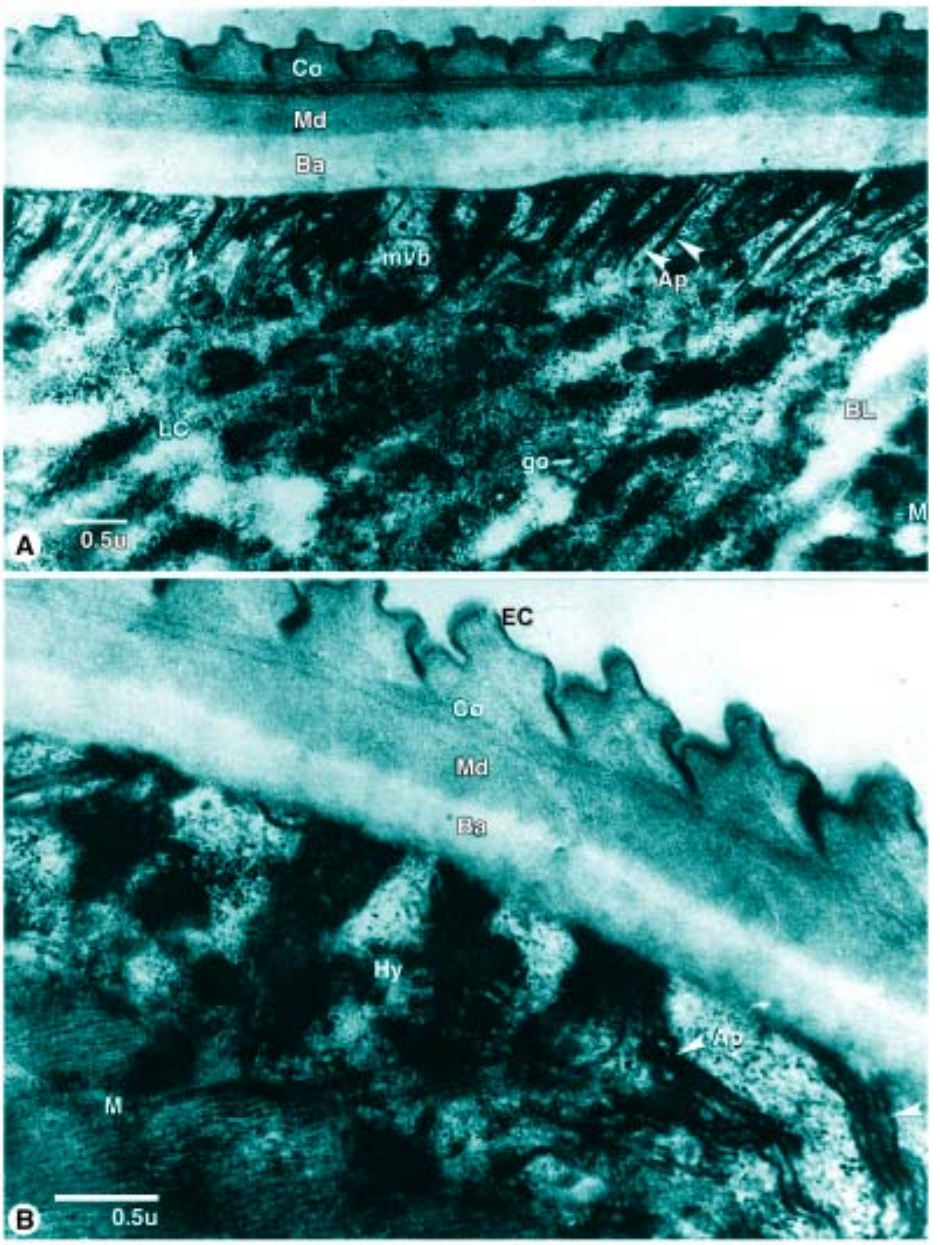

Fig 2 TEM components of the body wall of adult *B. pahangi*: the cuticle, lateral cord (LC), hypodermis (Hy) and somatic musculature (M). A) The cuticle is divided into three layers: cortical (Co), middle (Md) and basal (Ba). The lateral cord (LC) and muscle (M) are separated by a rather thick basal lamina (BL). The lateral cord cell is rich in infoldings of the apical plasma membrane (Ap) with mitochondria (mt) and multivesicular bodies (mvb) located in close relation to these apical infoldings. In the deeper part of the cell, Golgi bodies (go) can be clearly identified. B) Higher magnification showing the epicuticle (EC), an outer covering of the cuticle with the interrupted trilaminate membrane-like structure. The hypodermis (Hy) is a thin sheet of hypodermal cell that also bears apical infoldings of the plasma membrane (Ap).

layer is a very thin osmiophilic layer about 0.15 mm thick and is termed the "epicuticle" (Fig 2B). At higher magnification (Fig 2B), it appears as a trilaminate membrane that is periodically interrupted. The external cortical layer lies immediately underneath the epicuticle, and is terminated at the base of each groove between the annulation (Figs 1A, 1B, 1D, 1E, 2A, 2B). It is highly filamentous in structure and the filaments are arranged randomly in several directions (Figs 1C, 1F). The internal cortical layer (Figs 1C, 1F) lies at the base of the annulations, and appears as a dense layer of parallel filamentous structures, comprising about 2-5 narrow parallel sublayers, not all of which are clearly discernible (Figs 1, 2). Most filaments run parallel and oblique to the long axis of the body.

The middle layer comprises fine filamentous structures that are arranged in parallel. In a longitudinal section, the filaments appear as fine granules (Figs 1A, 1B), but in a cross-section they appear as closely packed parallel bundles (Figs 1C, 1F), which indicates that these filaments in the middle layer encircle the worm's body.

The basal layer is subdivided into two sublayers, the outer basal and inner basal layers with filamentous components of both sublayers are arranged in opposite directions. Filaments of the inner basal layer appear circular, while those of the outer basal layer are longitudinal (Figs 1C, 1F). The filaments of both basal sublayers are more delicate, shorter and smaller than those located in the more superficial layers. There is no evidence of a basal lamina separating the cuticle from the underlying hypodermis, and thus the inner basal sublayer of the cuticle is in direct contact with the plasma membrane of the hypodermal cells (Figs 2A, 3), however, in some sections hemidesmosomes can be observed (Fig 4).

Cuticle of the infective stage larva (L_3)

The cuticle of this stage is much thinner than that of the adult worm and various layers are less developed. The outermost covering is an electrondense osmiophilic layer that resembles the epicuticle of the adult, but it appears denser and is continuous throughout (Figs 5, 6). The cuticle comprises finer fibrils arranged randomly. No clear pattern is observed in the cuticular matrix at this stage of development. The hypodermis lies beneath the cuticle without a basal lamina separating them (Fig 7).

Hypodermis and cords

The hypodermis lies between the cuticle and the somatic musculature. It is a particularly important and metabolically active part of the nematode's body walls, being responsible for synthesis and maintenance of the cuticle. It is characteristically thickened in four regions around the cross-section of the body, forming a dorsal, a ventral and two lateral hypodermal cords. These cords protrude into the pseudocelomic cavity between the somatic muscle and divide the latter into four quadrants. The two largest projections are the lateral cords, which commonly run the whole length of the body. The dorsal and ventral cords are pedunculated parts of the hypodermal cells that also contain neural elements. Overlying the muscle in each quadrant (Fig 8), the hypodermis is only a very thin syncytial sheet formed by the extensions of cells whose soma are in the cordal regions (Fig 9).

In adults, the lateral cords (Figs 1A, 2A, 3A, 3C) are of variable thickness and protrude into the pseudocele, from which they are separated by a distinct basal lamina. Each lateral cord appears cellular and comprised of a small median cell enclosed between a pair of larger sublateral cells (Figs 1A, 8A, 8C); plasma membranes of adjacent cells cannot be readily identified in adults. The sublateral cells are separated from the muscle cells by a homogenous intercellular matrix of variable thickness. Basally, this matrix merges with the basal lamina. The cell cytoplasm is highly complex and is roughly divisible into three zones. The narrow subcuticular region containing arrays of infoldings (Figs 2, 3), which are invaginations of the apical plasma membrane. The broader midregion contains a nucleus, cell organelles and abundant glycogen. The innermost region contains a network of infolded basal plasma membrane (Figs 3B, 3C). Each apical infolding consists of invaginated double trilaminate and several folds appear aligned in parallel in one group. Arrays of folds are observed along the

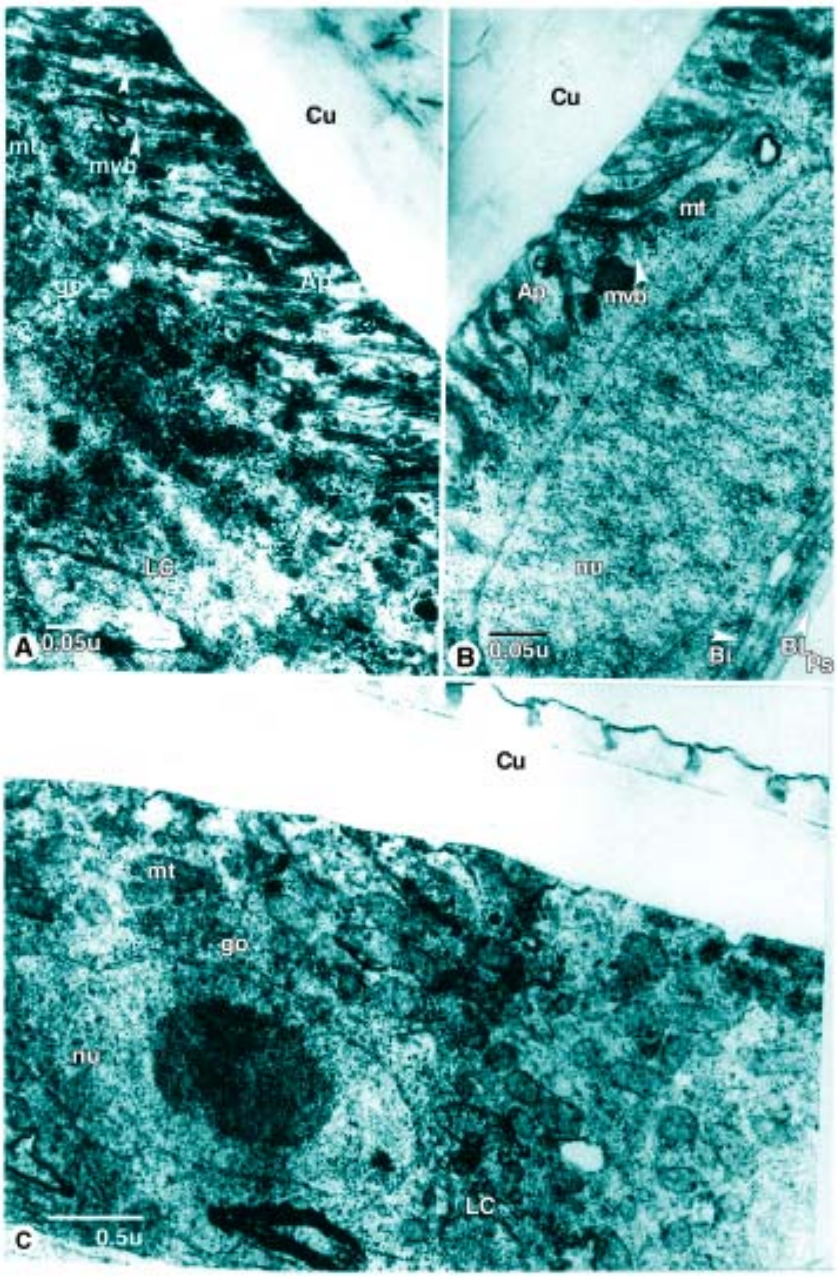

Fig 3 TEM of the lateral cord (LC) of adult *B. pahangi*: A-B) The cuticle (Cu) covers the lateral cord externally and the basal lamina (BL in B) lines it internally. The cell is divided into three regions: the apical region bears apical infoldings (Ap) with closely associated mitochondria (mt) and multivesicular bodies (mvb); the midregion contains the nucleus (nu) and other cytoplasmic organelles, eg, Golgi bodies (go) and glycogen deposits and the basal region has a network of infolded plasma membrane (Bi). C) Higher magnification of another part of the lateral cord (LC) showing the absence of apical infolding, although mitochondria (mt) are still abundant. The cell also shows euchromatic nucleus (nu) with prominent nucleolus and distinctive Golgi bodies (go).

whole apical surface (Figs 2B, 3B). Such apical infoldings are present throughout the surface area of the hypodermis. A number of mitochondria are present in the area close to the folds, and vary in size and shape. In the apical zone, there are also multivesicular bodies (Fig 2A) and numerous small vacuoles (Fig 3A). A large euchromatic nucleus, with a distinct nucleolus, is located in the midregion of the cell. Surrounding the nucleus are other organelles, such as the Golgi complexes, the rough endoplasmic reticulum (rer) and glycogen particles which appear as irregularly

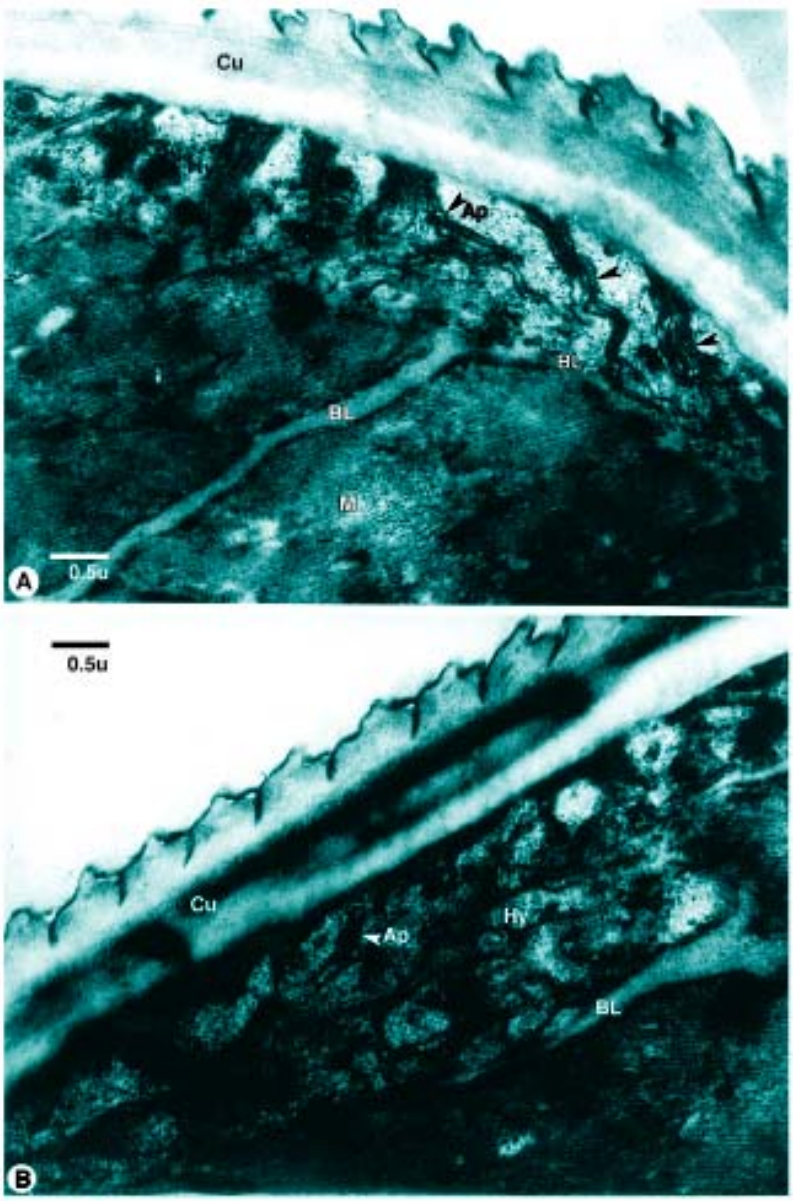

Fig 4 TEM of the intercordal region of the hypodermis (Hy) of adult *B. pahangi*: A-B) The scallop-shaped cuticle (Cu) covers a thin sheet of the hypodermis, which is separated from the musculature (M) by the basal lamina (BL). The hypodermal sheet (Hy) also bears distinctive series of parallel infoldings of the apical plasma membrane (Ap).

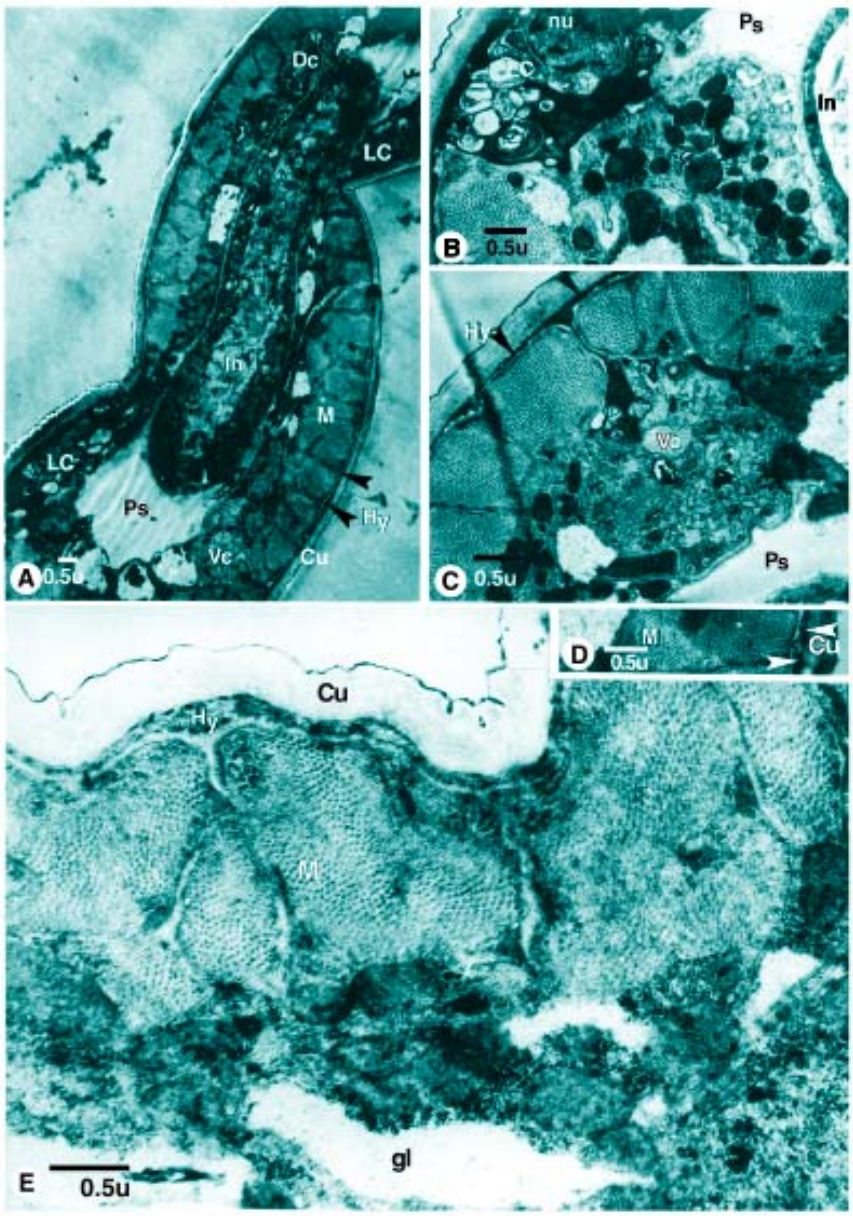

Fig 5 TEM of L_3 *B. pahangi*: A) The cuticle (Cu) covers the hypodermis (Hy), which is thickened to form 4 hypodermal cords: two broad lateral cord (LC) one dorsal cord (Dc), and one ventral cord (Vc); in the intercordal region, the hypodermis is only a thin sheet, and underneath are the somatic muscle cells. All the structures are lined by a continuous system of basal lamina and the intercellular matrix (IMa), which is rather thin in L_3, separating the body wall from the pseudocele (Ps). Only the intestine (In) occupies in the pseudocele of L_3. B) The lateral cord (LC) is composed of a hypodermal cell containing the usual organelles, such as Golgi complexes, and rough endoplasmic reticulum (rer). C) The ventral cord (Vc) is composed of part hypodermal cell and neural structure. D) The musculature (M) beneath the hypodermis (Hy) shows myofibrillar and non-myofibrillar portions, rich in glycogen (gl).

dispersed dark granules deposited in the unstained patches.

The hypodermis covering the somatic muscle cells is only a thin sheet of fused cytoplasmic processes of cordal cells. It is about 0.5 mm thick, and separated from the muscle by the basal lamina of variable thickness. This part of the hypodermal tissue also shows infoldings of the apical plasma membrane (Figs 4, 8). Other organelles present are small mitochondria, rer, ribosomes, multivesicular bodies and numerous small vesicles (Figs 9, 10)

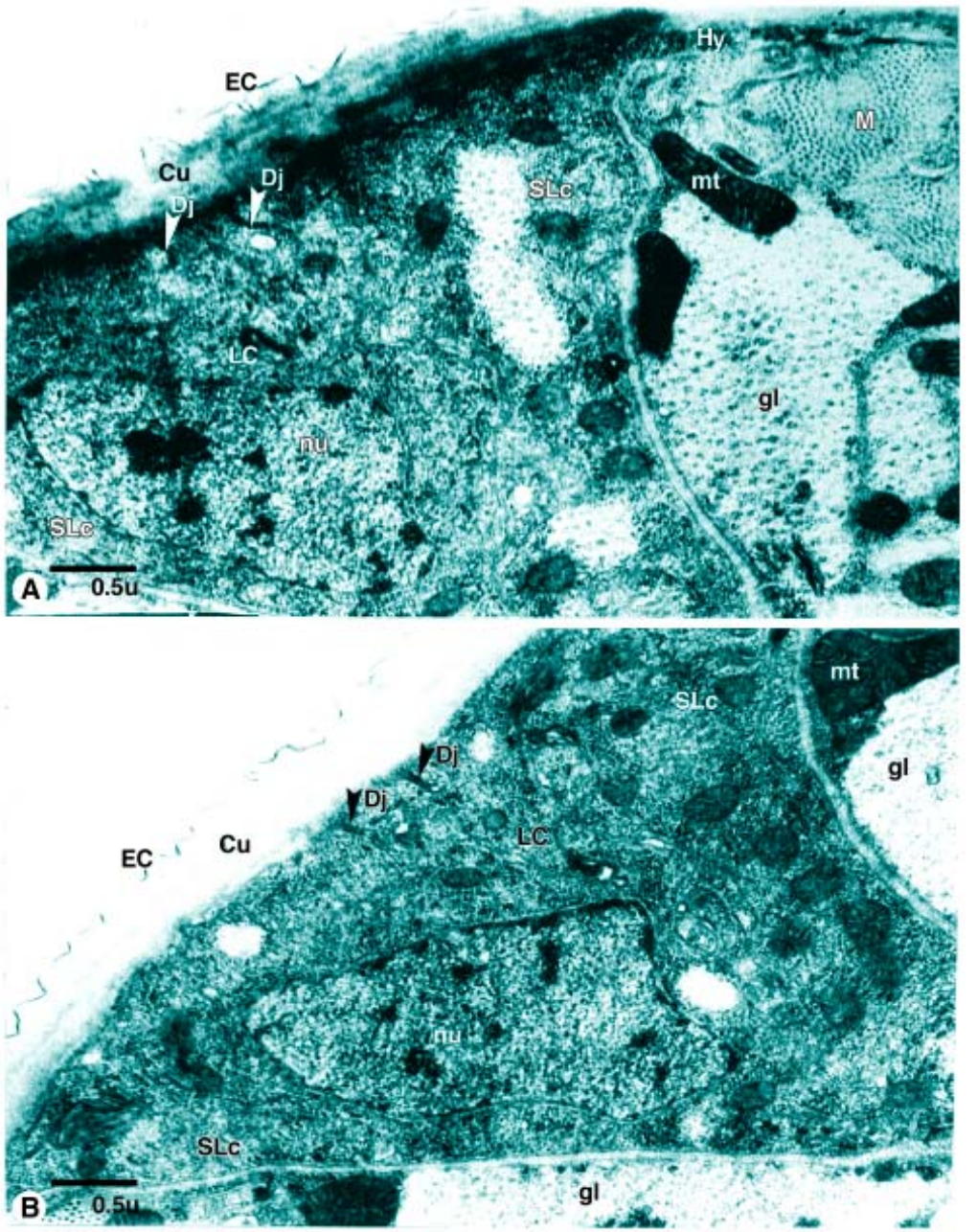

Fig 6 TEM of lateral cord of L_3 of *B. pahangi*: A-B) In some larvae, the plasma membrane between adjacent cells is difficult to define, except for the two desmosome junctions (Dj) located at their subcuticular region.

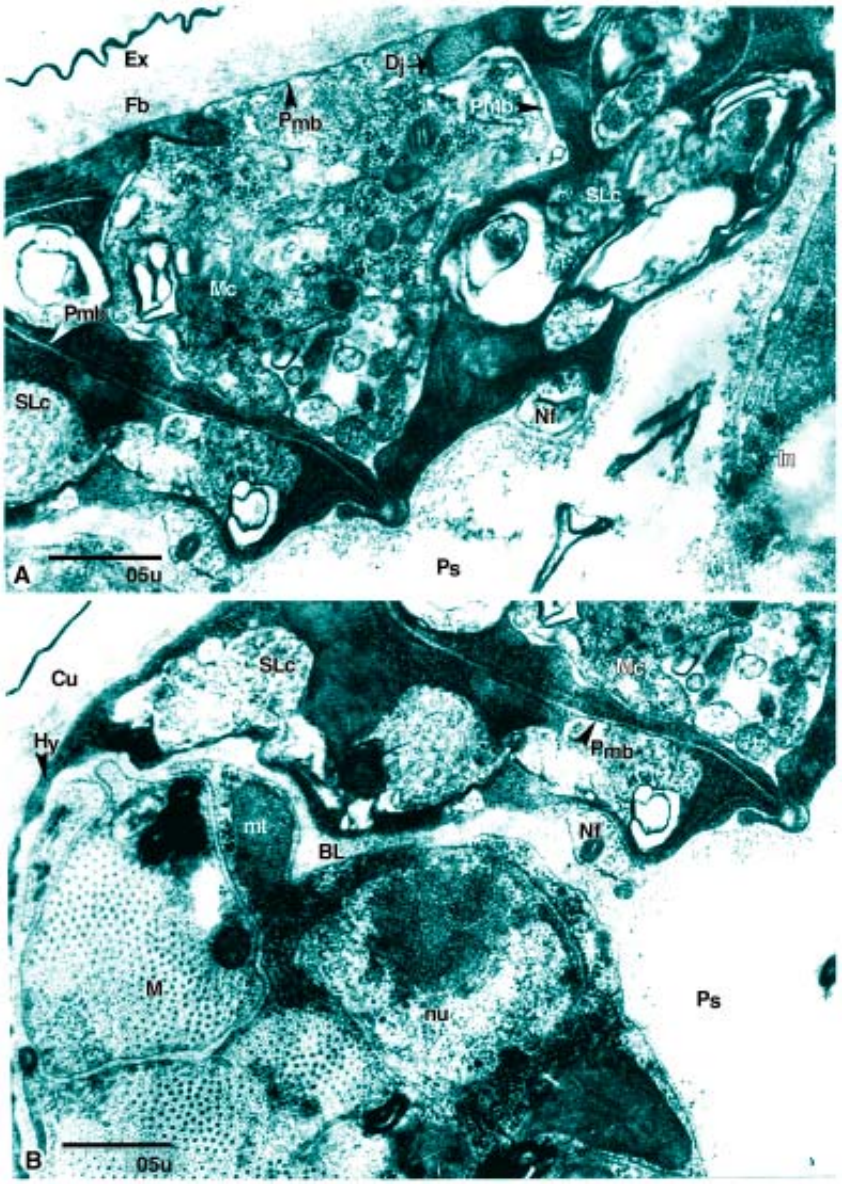

Fig 7 TEM of L_3 of *B. pahangi*: A) The cuticle shows a typically scalloped pattern, and is divided into an external homogenous layer (Ex), bound by an outermost osmiophilic layer and an internal fibrous layer (Fb) containing a fine fibrillar structure. The cuticle is separated from the hypodermis by the plasma membrane (Pmb) of the lateral cord cells. The lateral cord is composed of three distinctive cells: one small median cell (Mc) surrounded by two larger sublateral cells (SLc). Two desmosome junctions (Dj) joining these cells can be identified at the apical part. The plasma membrane (Pmb) between apposed cells can also be identified Nf = Nerve fibers are in close relation to the lateral cord. B) The sublateral cell (SLc) sends slender cytoplasmic process to become the hypodermal sheet (Hy) overlying the somatic musculature (M). BL = the basal lamina separates the lateral cord, and the hypodermis from the muscle, mt = mitochondria, nu = nucleus of muscle, Ps = pseudocele.

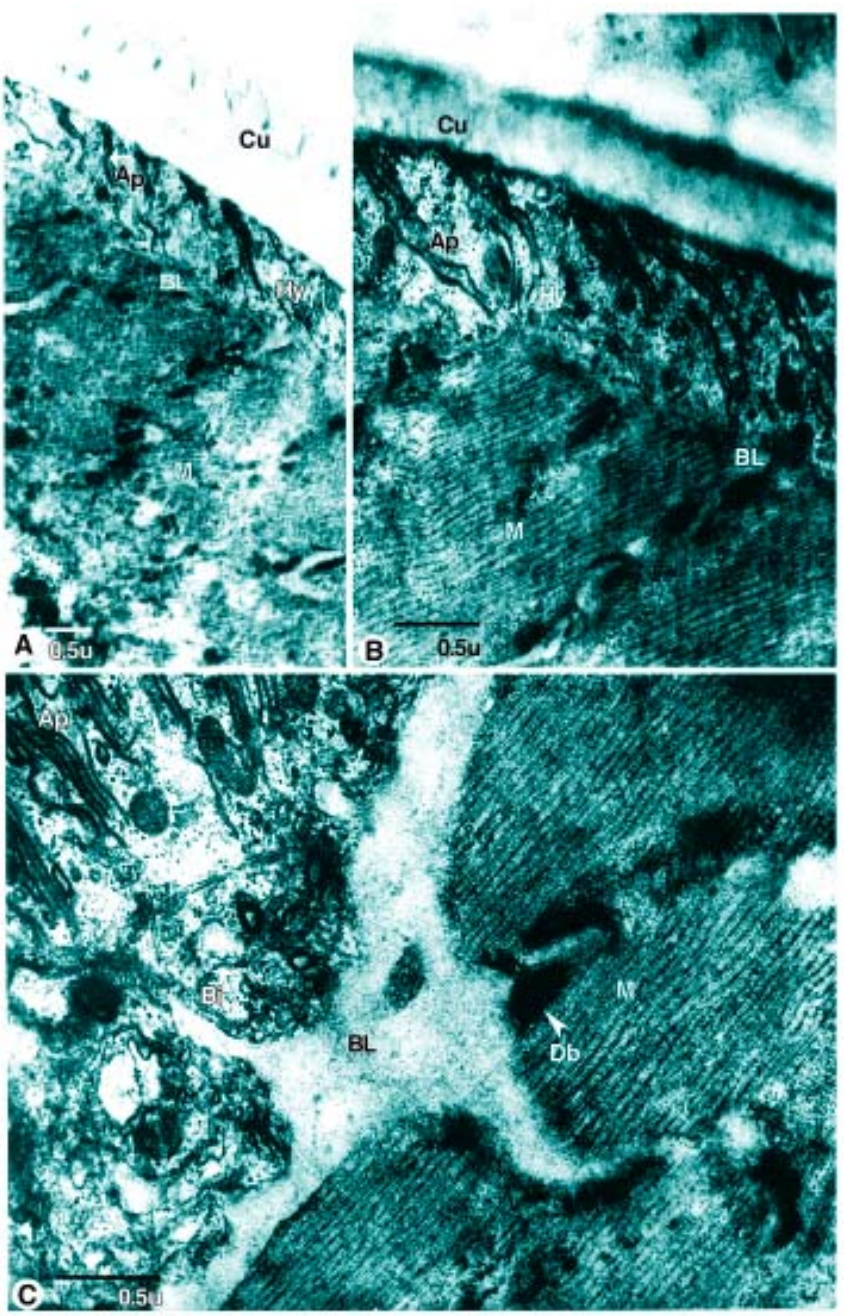

Fig 8 TEM of the intercordal region of hypodermis (Hy): A-B) The cuticle (Cu) covers the thin hypodermal sheet (Hy), which is separated from the muscle (M) by the basal lamina (BL). The hypodermis (Hy) has numerous apical infoldings (Ap). C) Higher magnification of the junction between the hypodermis and the musculature (M) that is separated by a homogeneous basal lamina (BL). The basal plasma membrane also has a stack of basal infoldings (Bi) but these are not as extensively developed as the apical infoldings. The muscle cell (M) plasma membrane has dense bodies (Db), which may serve as anchorage points for myofilaments and the basal lamina.

Discussion

The cuticle of the adult and L_3 of *B. pahangi* show no great structural variation, although the cuticle of L_3 is much thinner than that of the adult worms and various layers are less well-developed. All layers of the cuticle are composed of fibrils arranged in different directions, resulting in the division of this structure into five sublayers. This finding is similar to those reported by Roger *et al* [14] and Howells [15]. The cuticular protein of filarial worms was suggested by Philipp *et al* [16] and Selkirk *et al* [17] to be collagen-like, and covalently linked via disulphide bonds. Such proteins are believed to be the major components of the basal, middle and inner cortical layers, while the non-collagen proteins are believed to be the principal components found in the outer cortical layer. Ultrastructural examination in the present study, however, indicated that the cuticle is composed of a finer fibril structure, and of smaller dimension than the true collagen fibers that exist in mammalian tissues. The largest fibers are situated in the outer cortical sublayer, and are only about 7-10 nm in diameter. An individual fiber does not show periodicity. Further work is, therefore, required to characterize the true biochemical nature of these cuticular fibrils. In any event, these cuticular fibrils are one source of antigens that can elicit antibody production in hosts, as it was shown that solubilized cuticular collagens of *Brugia* were precipitated by human antisera against *B. malayi, W. bancrofti* or *O. volvulus* [17].

The cuticle of the adult worm of *B. pahangi* is rather thick, and only the outermost layer of the cuticle, the epicuticle, is exposed to the host

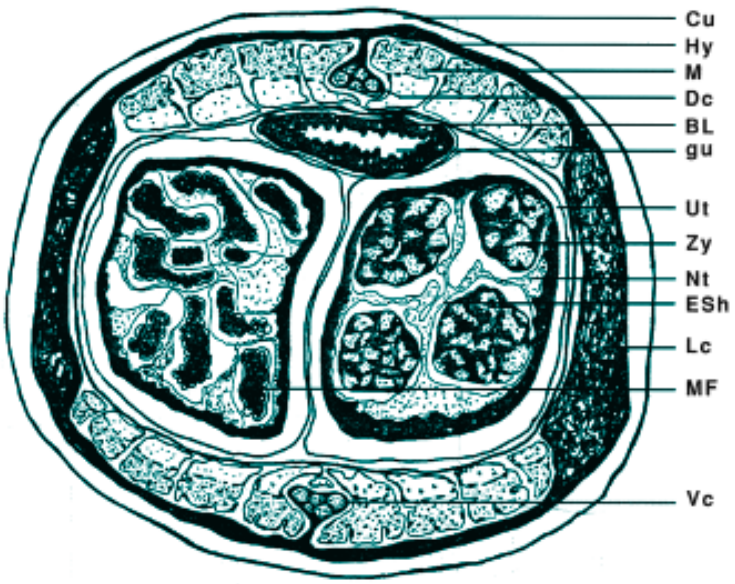

Fig 9 A schematic drawing of the midtransverse section of an adult female *Brugia pahangi* showing the cuticle (Cu) covering the hypodermis (Hy) and somatic muscle (M); these components of the body wall are separated from the pseudocele by a continuous layer of basal lamina (BL). The hypodermis is thickened at four hypodermal cords: two broad lateral cords (LC), one dorsal (Dc) and one ventral cord (Vc). The body cavity or pseudocele is occupied by the uterus (Ut) and the gut (gu) which are also surrounded by their own basal laminae. Depending on the level of the section, the uterus is filled with developing microfilariae (MF) or zygote (Zy) which are covered by the egg shell (ESh). These developing stages are bathed in the uterine fluid, which is colloidal in nature and may contain nutritive material (Nt).

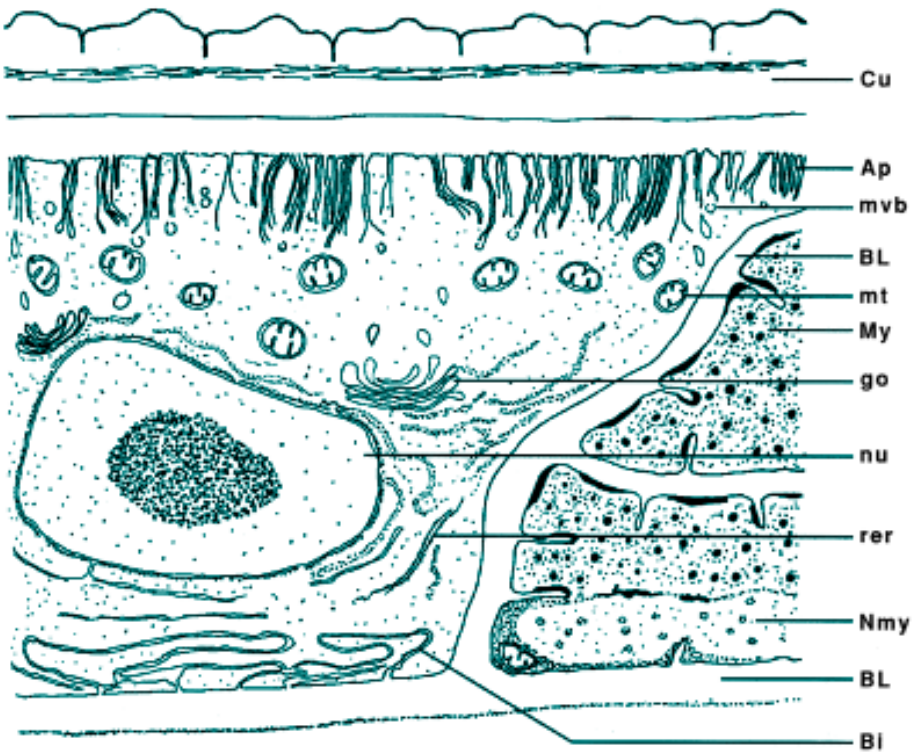

Fig 10 A schematic drawing of the lateral cord cell showing euchromatic nucleus (nu), abundant rough endoplasmic reticulum (rer) and ribosomes, mitochondria (mt), and well-developed Golgi complexes (go). The cell can be divided into three zones: the outer zone has numerous infoldings of the apical plasma membrane (Ap), at which the mitochondria (mt) and multivesicular bodies (mvb) are closely associated; the midregion contains the nucleus (nu) and other organelles, such as rough endoplasmic reticulum, and ribosomes. The basal zone is rich in basal infolds of the basal plasma membrane (Bi) which are not as extensive as the apical infoldings. A continuous line of basal lamina (BL) separates the body wall from the pseudocele. My = myofibrillar portion of muscle, Nmy = non-myofibrillar portion.

immune system. The epicuticle is quite unlike a plasma membrane because it lacks intramembranous particles [18-19] in the fractured planes between its exterior surface and the outlying surface coat. Although the cuticle was initially conceived as a tough, essentially inert layer, bound externally by the epicuticle, whose main function was to resist any lytic effects by host immune responses, more recent evidence has indicated that the cuticle may also possess other properties, such as molecular transfer across this layer as illustrated by the uptake of labeled amino acids via the cuticle of *B. pahangi* [20]. Fragmentary knowledge of the topography and composition of the cuticular proteins, and the epicuticle, limit our understanding of the dynamic properties of the surface. Since this structure may be one of the most important target of host immunity, it needs to be further studied, particularly its biochemical nature.

The hypodermis lies between the cuticle and the somatic muscle. It is an important and metabolically active part of nematodes, being responsible for secreting and maintaining the

cuticle. The surface area of the hypodermal cell is increased by two modifications: the apical membrane infoldings form series of lamellae that run in parallel arrays; and the basal membrane infoldings, which become highly elaborate in the lateral cords. The increased outer membrane may facilitate the exchange of materials with the cuticle, particularly the secretion of cuticular components from the cytoplasm of the hypodermis. The increased inner membrane may also enhance exchange of materials with muscle cells and the inner compartments of the parasite's body.

The nuclei are situated in the lateral cord cells. They are surrounded by rer, ribosomes and Golgi bodies. These organelles are also present in the intercordal hypodermis, which implies the presence of synthetic activity throughout the layer. Mitochondria are distributed throughout the hypodermis, but more are found in close association with the infoldings of the outer membrane zone, where there are also multivesicular bodies with microtubules around this region. Microtubules found in this area may facilitate the movement of these vesicles to the apical cell membrane, or apical infoldings, where they are exocytosed to release the materials to form the cuticle. Furthermore, the hypodermal sheet is probably responsible for the exchange of materials, eg, nutrient molecules from the environment, and the excretion of waste materials through the cuticle into the environment. Glycogen deposits and lipid droplets are scattered throughout the cytoplasm. This characteristic is similar to what Wright [21] had been reported in *B. malayi*, and also suggested that the distribution of glycogen represented the polarization of metabolic activity within the cells. The infoldings of the plasma membrane of the basal part of the cells associated with the multivesicular bodies, mitochondria and small membrane bound vesicles also indicate the possibility of exchanges of materials across the cuticle. Pease [22] first called attention to the similarity between these infoldings and their associated mitochondria with the epithelial cells of mammalian distal convoluted tubules, ocular ciliary body and submandibular salivary gland, whose roles are water and ion transport.

Summary

Transmission electron microscopy revealed that the cuticle of adult worms and L_3 of *B. pahangi* show no great structural variation, although the cuticle of L_3 is much thinner and the layers are less well-defined. All layers are composed of fibrils arranged in different directions. The outermost layer, the outer cortical layer, is surrounded by a discontinuous trilaminate membrane-like epicuticle which is one of the richest sources of antigens that stimulate antibody production in hosts. The cuticular fibrils are believed to be collagen-like proteins, comprising fibrils finer than the true collagen fibers, and the individual fibril shows no periodicity. The cuticular proteins may be synthesized and secreted by the underlying hypodermis. This cuticular component may be slowly turned over and be released to the environment and act as one source of immunogens that can induce a host's production of antibodies.

The lateral cord cells of the hypodermis show distinctive zonation of the cytoplasm: the apical infoldings of plasma membrane, the midregion containing a large euchromatic nucleus, glycogen and organelle characteristics of highly synthetic activity, and the basal region with its network of basal infoldings. The hypodermis is lined with a thin basal lamina, which is a continuous system, separating the body wall from the pseudocele. In addition to its role in synthesizing cuticular proteins, the infolded plasma membrane of the hypodermal cells may play roles in controlling and facilitating the exchange of materials through the cuticle to the environment, for both nutritive, excretory and osmoregulatory purposes.

Acknowledgements

The authors would like to thank Dr Wanlop Chusattayanond, Department of Microbiology, Faculty of Science, Mahidol University who kindly gave all the parasite specimens.

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