THE ULTRASTRUCTURE OF THE GRAVID UTERUS OF *BRUGIA PAHANGI*, ANOTHER RICH SOURCE OF ANTIGEN OF THE FILARIAL PARASITE

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Abstract. The gravid uterus with zygotes and microfilariae *in utero* of *Brugia pahangi*, a rich source of antigen as revealed by a recent immunofluorescent technique, were studied ultrastructurally. The epithelial cells of uterus show ultrastructural features of synthetically active cells. Their secretions may provide nutrients for the egg *in utero.* On the basal side, the uterine epithelial cells may also secrete substances to form the basal lamina of the uterus which is rather thick and irregularly fused with the basal lamina lining the body wall where the pseudocoelomic cavity is obliterated. For the most part, the uterine basal lamina contains uniform granular material of moderate electron density. There are also elongated visceral muscle cells embeded in it, and which surround the uterus, with adjacent cells overlapping. The gravid uterus contains several stages of developing microfilariae within its lumen, the cleaving zygotes are also present at another level. The morula of zygotes are composed of several closely packed cells surrounded loosely by their own egg shell membranes. The egg shell becomes more convoluted as development proceeds. The egg shell surrounding the developing microfilariae *in utero* is secreted by the uterine epithelium. This structure later becomes the sheath of circulating microfilariae, and is highly antigenic as indicated by intense labeling with fluorescent antibodies.

INTRODUCTION

Filariasis is a group of human and animal diseases caused by arthropod-borne nematode parasites of the Order Filariidea. Eight of these species are found to produce disease in humans, they are: *Wuchereria bancrofti, Brugia malayi, Brugia timori, Onchocerca volulus, Loa loa, Dipetalonema streptocerca, Mansonella ozzardi* and *Mansonella perstans*.

Lymphatic filariasis in humans is caused by the developing and adult forms of filarial parasites present in the lymphatic system. Three parasites belonging to two genera are responsible, including *W. bancrofti, B. malayi* and *B. timori* (Mak and Dennis, 1985). While *W. bancrofti* is found throughout the wet tropical world, *B. malayi* is confined to Asia and *B. timori* is only found on the islands of Timor, Flores, Alar and Roti in Indonesia (Wheeling *et al*, 1975). In Thailand, *W. bancrofti* is found in the western part of the country along the border with Myanmar, while the endemic area for *B. malayi* is in the south.

It has been reported that the control program for filariasis in southern Thailand had been successful (Harinasuta *et al*, 1981), but a report covering 1982-1985 by the Filariasis Division, Ministry of Public Health of Thailand, including areas previously inaccessible to government health personnel revealed new areas of high endemicity. In Thailand, this underestimation is also true due to underreporting and a relative limitation of personnel and unavailability of proper diagnostic tools for field surveys, which depend primarily on the detection of microfilariae on thick blood film. The fact that blood samples need to be taken at night when the microfilariae appear

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in the circulation is a major hindrance in the detection and control program. Attempts at serological tests are aimed at antibody detection, which usually appears late and does not allow detection of early (premicrofilaremic stages) infection when chemotherapy can be more effective and transmission can be interrupted. Improved immunodiagnostic tests, particularly those aimed at the detection of early stages of parasite antigens are thus required. Previous attempts have met with the problem of the complexity of the parasites. Therefore, careful studies, using modern techniques to dissect the antigenic structures of the filarial parasite, are necessary. The ultrastructure and the immunocytochemical studies which aim at the demonstration and localization of the antigenic sources should also be one important area that will complement such a study. Since they will provide better understanding of the nature and function of several organs of the parasite that are the sources of antigens.

Brugia pahangi, a common filarial parasite of wild and domestic animals in Southeast Asia (Laing *et al*, 1960; Mak *et al*, 1980; Lim *et al*, 1984; Palmieri *et al*, 1985), occurs naturally as a parasite in cats, where the adults parasitize the lymphatics, and the microfilariae circulate in the blood. Thus, the *B. pahangi* infected cat is a good model for various laboratory studies of human filariasis. Since the animal infection with *B. pahangi* is easier to control and manage than the other two species of human filaria, it is imperative to prove whether *B. pahangi* is antigenically closely related to the human lymphatic filarial worms, *B. malayi* and *W. bancrofti*. The cross-reactivity of antigens and the protective effect, if proved to be substantial, could provide a comparatively convenient means of producing antigens from easy-to-culture *B. pahangi* instead of those from the difficult-to-culture human filariae. Furthermore, gene cloning techniques may be applied to produce *B. pahangi* antigens in large amounts that can be used for immunodiagnosis as well as vaccination purposes instead of antigens from human filaria species.

Recently the antigenic sources of the adult and third stage larvae (L_3) of *B. pahangi* were detected by indirect immunofluorescent technique, using six panels of antisera, including human antisera against *B. malayi* and *W. bancrofti*, cat antisera against *B. malayi* and *B. pahangi* and jird antisera against *B. malayi* and *B. pahangi* as primary antibodies (Roongruangchai *et al*, 2003). All antibodies giving the same results indicate non-species specificity and that *B. pahangi*, *B. malayi* and *W. bancrofti* must share most of the comon antigenic molecules. The most intense fluorescence was located at the epicuticle, basal lamina of the body wall, basal laminae of the gut, reproductive tracts, egg shell *in utero* and sperm. Since the gravid uterus with the egg shell of the microfilariae *in utero* had been demonstrated to be a highly antigenic source, the purpose of this study was to demonstrate the ultrastructure of the gravid uterus with the zygotes and microfilariae *in utero* for better a understanding of the structure and function, and to propose a reason for the high antigenicity.

MATERIALS AND METHODS

Specimen collection

The adult worms were recovered from the peritoneal cavities of jirds *(Merione unguiculatus*), which had been previously infected by injecting the infective stage larvae into the peritoneal cavities 60 days earlier by the method of McCall *et al* (1973). The jirds were sacrified and the abdominal cavities were exposed by making a small incision line, and the adult worms were collected by two pairs of tweezers, and washed several times in PBS.

TEM preparation

For the ultrastructural studies the adult female worms were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 at 4ºC 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 4ºC for one hour and washed three times with distilled water. After tertiary fixation and staining in 1% uranyl acetate in distilled water for 20 minutes, the parasites were washed three times with distilled water. They were dehydrated in a graded series of etha-

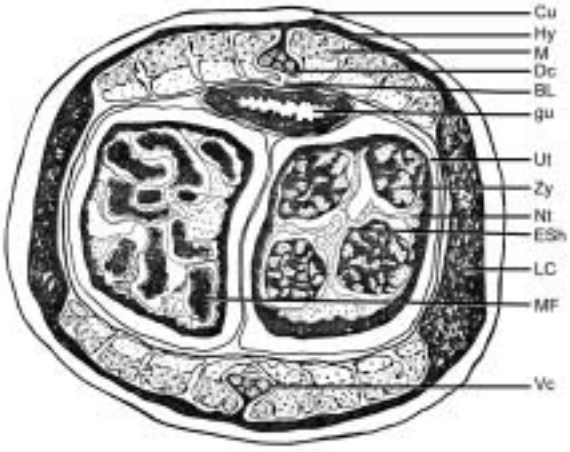

Fig 1–A schematic drawing of the midtransverse section of an adult female of *Brugia pahangi* showing the cuticle (Cu) covering the hypodermis (Hy) and somatic muscle (M); these components of the body wall are separated from the pseudocoel by a continuous layer of basal lamina (BL). The hypodermis is thickened at four hypodermal cords: two broad lateral cords (LC), one dorsal (Dc) and one ventral cord (Vc). The body cavity or pseudocoel 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 the zygotes (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).

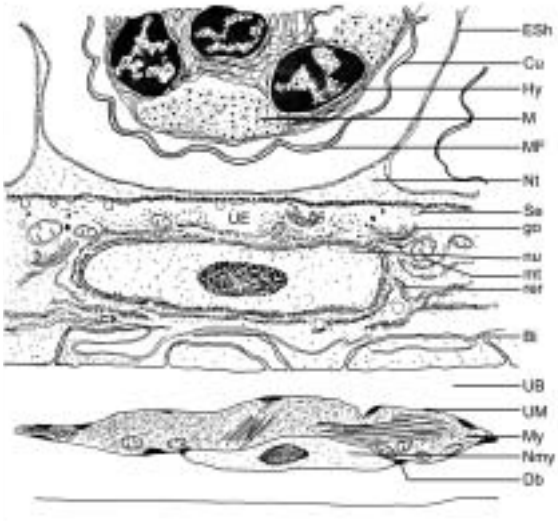

Fig 2–A schematic drawing of the uterine wall comprising uterine epithelial cells (UE) bound by a thick basal lamina (UB) in which the uterine muscle cells (UM) are embedded. The microfilariae (MF) are covered by loosely fitting egg shell (ESh) and they occupy almost the entire uterine lumen. The uterine epithelial cell contains a large euchromatic nucleus (nu), an extensive system of rough endoplasmic reticulum (rer), Golgi bodies (go) and abundant tiny secretory vesicles (Se) that are secreted at the luminal surface. The secretory material appears to contribute to form the component of egg shell and nutritive material (Nt). The cell also has an extensive network of basal infoldings (Bi) at the basal surface. Each uterine muscle cell is comprised of two major parts: the myofibrillar (My) and non-myofibrillar (Nmy) portions. The former contains thick and thin myofilaments anchored to the dense bodies (Db); the latter comprises other organelles, particularly nucleus, mitochondria (mt). The developing microfilariae show scallop-shaped cuticle (Cu) lined by a denser layer of hypodermis (Hy) and clearly identified muscle cell (M) with thick and thin myofilaments. The interior of the body appears to be still undifferentiated and contains densely packed cells with heterochromatic nuclei.

nol (50% to 100%) for 15 minutes at each step and then infiltrated twice in propylene oxide for 20 minutes each time at room temperature. The solution was replaced twice with the mixture of propylene oxide and araldite plastic at the ratio 2:1 for one hour at room temperature and a 1:2 mixture overnight at room temperature, then they were embedded in Araldite plastic. The plastic was allowed to polymerize at 37ºC for one day then at 45ºC for two days and 60ºC for three days. Thin sections showing silver to gold interference were mounted on formwar coated copper grids and further stained with uranyl acetate and lead citrate for 30 minutes each and observed by transmission electron microscope.

RESULTS

The uterus is the longest part of the female reproductive tract. Its wall comprises epithelial cells surrounded by a thick basal lamina (Figs 1, 2, 3A, 3B, 4A, 5A, 6, 7B and 8) whose outer part contains visceral muscle cells. Each epithelial cell has a large euchromatic nucleus with one distinct nucleolus. The cytoplasm comprises an extensive system of rough endoplasmic reticu-

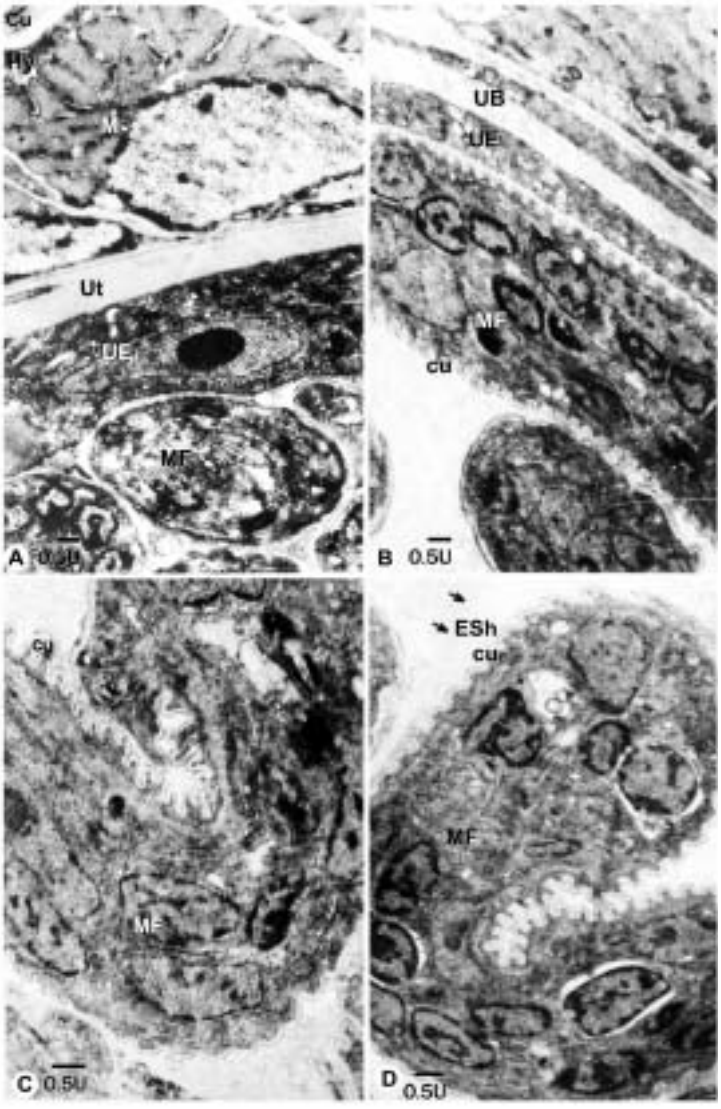

Fig 3–A TEM of adult female of *B. pahangi* uterus with microfilariae *in utero*: A) The body wall is comprised of cuticle (cu), hypodermis (Hy) and somatic musculature (M) lined by basal lamina which is apposed to the uterus (Ut). The pseudocoel is obliterated by compression of the gravid uterus. The uterine epithelial cells (UE) show prominent euchromatic nucleus with fimbriated luminal surface. The uterine cavity contains developing microfilariae (MF). B) Higher magnification of the uterus with microfilariae *in utero*: the uterine wall is composed of epithelial cells (UE) lined by a thick basal lamina (UB). The microfilaria (MF) shows an extensive nuclear column bound externally by the cuticle (cu) which shows distinctive annulations. The microfilariae are bathed in a granular or floccular matrix, suggesting that it is a colloid in the living state. C-D Higher magnification of individual microfilariae *in utero* showing an extensive nuclear column occupying the whole interior of the body, which still lack pseudocoel at this stage. The scallop-shaped cuticle is bound externally by an osmiophilic layer. The egg shell (Esh) surrounds each microfilaria (MF).

lum (Figs 3A, 4A), mitochondria and Golgi bodies. The basal aspect of the cells which faces the basal lamina bears basal infoldings (Figs 2, 4A), while the luminal aspect has a secretory characteristic with released substance, appearing as fine electrondense granules making linear contact with the apical membrane (Figs 2, 4A, 7B, 8). This substance may contribute to the egg shell. The epithelial cells also show evidence of exocytosis with omega-shaped invaginations of the apical cell membrane (Figs 2, 5A). The basal lamina of the uterus is rather thick, and in it are embedded elongated visceral muscle cells that also surround the uterus, with adjacent cells overlaping (Figs 2, 3A, 3B, 4A, 5, 6). The inner part of the muscle cells contains typically thick and thin myofilaments that anchor at the dense bodies situated along the cell membrane. The outer part of the cells contains other cytoplasmic organelles, the nucleus and mitochondria. The outermost part of the basal lamina is bound by an electrondense line that separates it from the basal lamina of the body wall (Fig 5).

The gravid uteri contain several stages of developing microfilariae within their lumen (Figs 3-4, 6-8). Following fertilization, each zygote develops prominently ruffled membranes. Later, the morula of zygotes are composed of several closely packed cells surrounded loosely by their own egg shell membranes (Fig 2, 3D, 4, 6-8). The space between the zygotes and the egg shell also contains

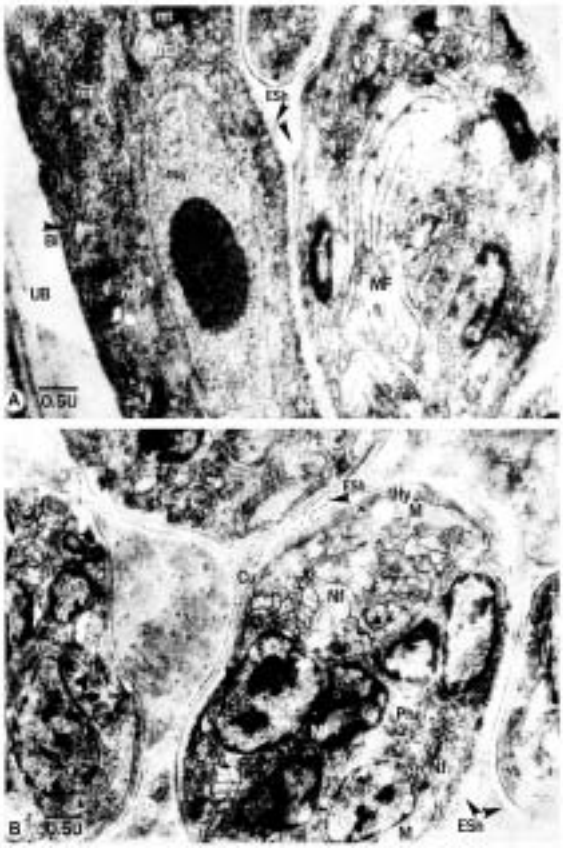

Fig 4–A TEM of adult female of *B. pahangi*: A) High magnification of the uterine epithelial cell (UE) which rests on a very thick uterine basal lamina (UB). Each epithelial cell has a large euchromatic nucleus (nu) with one distinct nucleolus. The cytoplasm comprises an extensive system of rough endoplasmic reticulum (rer), and mitochondria (mt). The basal part of the cell has basal infoldings (Bi). At the luminal surface of the cell, the secreted egg shell (Esh) of a microfilaria (MF) is still attached to it, appearing as fine electrondense granules. B) High magnification of fully developed microfilariae (MF) still *in utero.* The cuticle (Cu) is merely a thin homogenous layer covered by 2 parallel electrondense layers. The hypodermis (Hy) separates the cuticle from the muscle cell (M). The interior of microfilaria contains extensive neural elements rich in bundles of nerve fibres (Nf). The pharyngeal tract (Ph) is still small in diameter surrounded by six to nine small cells at the region of nerve ring. At this stage of development, the egg shell (Esh) is stretched loosely around microfilariae and is highly folded (arrow).

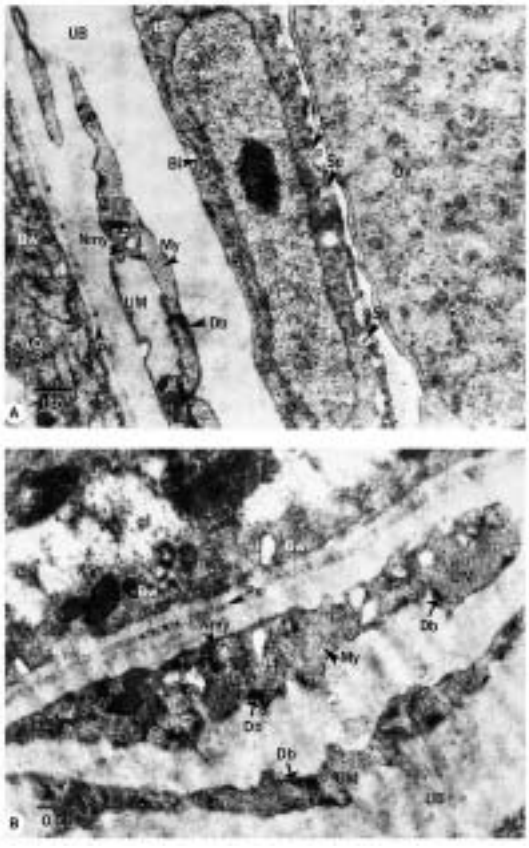

Fig 5–TEM of female of *B. pahangi*: A) The uterine epithelial cell (UE) showing large euchromatic nucleus (nu) with a distinct nucleolus, basal infolding of the plasma membrane (Bi). The luminal surface of the cell shows exocytosed secretory granules (Se) towards the lumen which contains fertilized ova (OV). In the basal lamina (UB) the elongated visceral muscle cells (UM) are embedded. Each muscle cell is comprised of a myofibrillar (My) and non-myofibrillar (Nmy) portion. The dense plaques (Db) serve as anchoring points for the myofilaments. B) Detailed morphology of uterine muscle cells (UM) surround the uterus, are embedded in the basal lamina (UB) and overlap each other. The interior contains typical thick and thin myofilaments (My) that are anchored to the dense body (Db) along the cell membrane. The non-myofibrillar part (Nmy) contains organelles, such as mitochondria (mt). The gravid uterus compresses the pseudocoel (Ps) against the body wall (Bw). Glycogen (gl) is abundant in the non-myofibrillar part. LC, lateral cord.

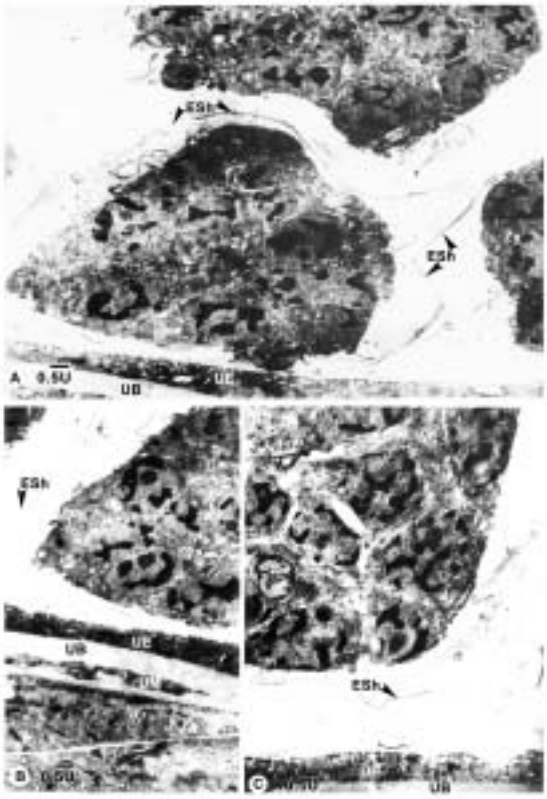

Fig 6–A TEM of the gravid uterus of *B. pahangi*: A-C) The cleaving zygotes (Zy) at the morula stage contain several cells in tight packing; the outermost cells show a ruffled border and are surrounded by a loosely fitting egg shell (Esh). The space between the zygote and the egg shell also contains colloidal material which could have nutritive function. The egg shell and nutritive material are presumably secreted by the uterine epithelial cells (UE) which are highly stretched into thin cells in close apposition to the newly secreted egg shell. UB, basal lamina; UM, uterine muscle cell.

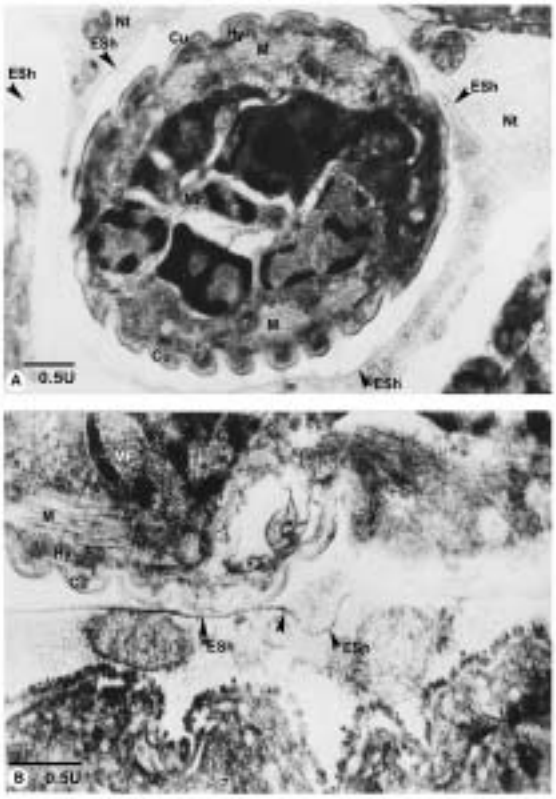

Fig 7–A TEM of *in utero* microfilaria of *B. pahangi*: A) The more developed microfilaria (MF) *in utero* shows a very thin cuticle (Cu) bound by 2 osmiophilic layers that apear to be discontinuous at the point of deepest indentation. The hypodermis (Hy) separates the cuticle from the musculature (M) which contains thick and thin myofilaments. The interior of the body is filled with the nuclear column. The microfilaria is bound by the loosely fitting egg shell (ESh), that is, inturn, surrounded by an electrondense granular matrix which may be nutritive material (Nt). B) Higher magnification showing the detailed ultrastructure of the egg shell (Esh) which is a uniformly thin layer sandwiched between an outer and inner particulate layers of greater electron density. The outer particulate layer appears as an irregular, fenestrated electrondense network over the surface of the sheath. The microfilaria (MF) is cut longitudinally and shows the annulation of the cuticle (Cu). Hypodermis (Hy) is still a very thin and hardly discernable layer, while cut longitudinally muscle cells contain both the thick and thin myofilaments (M). The uterine epithelial cell (UE) shows a fimbriated apical surface with fine electrondense granules on it (black stars).

granular material, that is probably a nutrient substance secreted by the uterine epithelial cells to nourish the zygotes. As the development proceeds, the egg shell becomes more convoluted and adjacent egg shells come into close contact with each other, and with the uterine wall (Fig 6A, B) where the egg shells of embryos nearest the uterine wall (Fig 7) are in intimate contact with the electrondense material coating the apical surface of the uterine epithelial cells (Figs

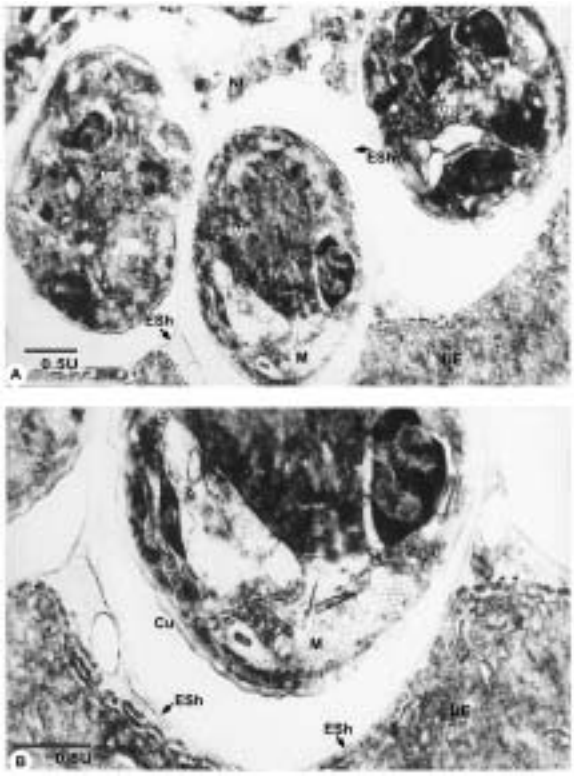

Fig 8–A TEM of the uterus of *B. pahangi*: A) Microfilariae (MF) *in utero* are surrounded by loosely fitting egg shell (ESh), the space between which is filled with electrondense nutritive (Nt) granules. The egg shell and nutritive material (Nt) are probably secreted by the uterine epithelial cell (UE). B) Higher magnification showing the apical part of a uterine epithelial cell (UE) revealing the dark dots along the margin of the cell (black stars) that probably become the egg shell and nutritive material. The microfilaria in the center of this micrograph shows the beginning of the cuticle (Cu) which is bound at the outermost part by an osmiophilic layer that is composed of three electrondense membranes, but in some preparations it consists of only two layers.

7B, 8). The embryos are so closely packed that their egg shells come into close apposition to the uterine epithelium (Fig 6A, 7B, 8) leaving only narrow channels in which electrondense material, similar to that secreted by the cells of the uterine wall, can be seen. The space between the embryo and the egg shell also contains scattered granular material (Figs 3D, 6-7).

The egg shell consists of a uniform central layer sandwiched between an outer and inner particulate layers of greater electron density (Figs 7, 8). The outer particulate layer of the egg shell appears as an irregular fenestrated electrondense network over the surface. Particulate materials also adhere to the inner side of the egg shell and give the appearance of an electrondense layer.

As development proceeds, the embryos become more compact, taking a C-shaped form (Fig 3C, D). By this time, the cuticle has formed, embryos are less crowded and each is well separated from its greatly extended and convoluted egg shell. However, the egg shells of embryos adjacent to the uterine wall remain in close apposition to the epithelial cell surface, and the space between egg shells of adjacent embryos becomes larger and filled with electrondense material (Fig 8). The egg shells are always larger than the embryos they cover and are often deeply folded (Figs 6-8). When embryos are fully elongated and mature, the egg shells are stretched loosely over them, and the embryos become detached from the uterine wall and from the neighboring embryos.

The cuticle of the fully developed microfilariae *in utero* or in the blood circulation appear to be similar. The cuticle is divided into outer and inner layers, and cuticular annulations are clearly seen in longitudinal sections (Figs 3B-D, 7, 8B). The outermost layer of the cuticle is very dense (Figs 3B-D, 7) and shows a break in continuity at the point of the deepest cuticular indentation and it is clearly trilaminate (Figs 7B, 8B). The inner layer of the cuticle is thicker, more electronlucent and appears rather homogenous with a very fine granulated structure when observed at high magnification (Fig 7).

The hypodermis of the fully developed microfilariae *in utero* forms the middle layer of the body wall (Figs 2, 7). In transverse sections, it has four components: two of which are small, and each forming the central part of the lateral cord, the other two components form intercordal hypodermis. The hypodermis is generally very narrow but the nucleated regions bulge into the center of the worm (Fig 7).

The muscle cells of microfilariae are elongated and have a spindle shape, lying parallel

with the long axis of the worm (Figs 2, 4B, 7, 8). In transverse sections, the muscle cells are located in four quadrants immediately beneath the intercordal hypodermis (Fig 7A). The contractile portion is composed of thick myofilament, each surrounded by several thin myofilaments. The non-contractile portion contains other organelles and abundant glycogen patches.

The interior of microfilariae comprises the nuclear column, together with an irregular mass of fine, dense granules (Figs 3, 4B, 7). The largest cell, occupying an entire transverse section, is situated just posterior to the inner body wall. Apart from this very large nucleus, other cells show no unusual characteristics (Fig 7).

DISCUSSION

The presence of prominent rough endoplasmic reticulum, Golgi bodies and fimbriated luminal borders of the uterine epithelium of *B. pahangi* are similar to what have been reported by Vincent *et al* (1975) in *B. malayi*. These characteristics imply that these cells have a large capacity for synthesis and secretion of materials to nourish the fertilizing gamete cells and the developing microfilariae at the time of fertilization and thereafter. The present findings reveal that secretion at the luminal aspect of the cells may contribute to the egg shell formation and also provide nutrient materials for developing microfilariae *in utero*. As in the immunofluorescent study (Roongruangchai *et al* 2003), the egg shell as well as the luminal aspect of the uterine epithelium are intensely labelled, this implies that these layers are highly antigenicity.

Microfilariae, the first stage of developing filarial nematodes, acquire an encasing structure from the egg shell *in utero*, which in most species is retained after birth. The egg shell turns into the structure called the sheath, which is present in microfilariae of *W. bancrofti, Brugia* spp, *Loa loa* and *Litomosoides carinii*. Other microfilariae break out of the egg shell *in utero* and live in the host's tissues unsheathed, for example, in *Dirofilaria immitis* and *Onchocerca volvulus* (Sayers *et al*, 1984).

The sheath is an important source of filarial antigens because it is exposed directly to the host immunity. The carbohydrate nature of the surface of the sheath was suggested by positive staining with ruthenium red and lanthanum hydroxide (Chen and Howell, 1981). Furman and Ash (1983) extended the observations on the carbohydrate make-up of the sheath of *B. pahangi* by using fluoresceinated lectins, and found that the sheath and the epicuticle of *B. pahangi* microfilariae stained positively with concanavalin A, which suggested that it contains mannose and/or glucose residue. In addition, the sheath also showed activity for acid phosphatase, 5′ nucleotidase and peroxidase. Kaushal *et al* (1984) studied stage-specific expression of carbohydrates on *B. malayi* and found that the L_3 stage and the adult worms did not bind to any of the lectins, while the microfilariae, on the other hand, bound to wheat germ agglutinin. The binding of this lectin was saturable and specific and attributed to the presence of N-acetyl-D-glucosamine. Microfilariae derived *in vitro* also bound to concanavalin A, which indicated the presence of glucose and/or mannose on this stage of parasite. In contrast, the binding of concanavalin A was not observed on microfilariae recovered directly from infected hosts, indicated that there might be a masking or loss of parasite specific antigens as microfilariae matured *in vivo*. The microfilariae of *B. malayi* did express N-acetyl-D-glucosamine-containing molecules in a stage specific manner, while *B. pahangi* contained mannose and/or glucose on the sheath and epicuticle (Furman and Ash, 1983). These carbohydrate moieties on the sheath may be an important component of surface antigens.

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