Formation of basement membrane-like structure terminates the cellular encapsulation of microfilariae in the haemocoel of *Anopheles quadrimaculatus*

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SUMMARY

The encapsulation of microfilariae in the haemocoels of mosquitoes combines both humoral and cellular reactions: the microfilariae are first encased in an acellular layer of melanin, followed by a cellular encapsulation by plasmatocytes. In this study, we demonstrated that cellular encapsulation of *Brugia pahangi* microfilariae in the haemocoel of the mosquito *Anopheles quadrimaculatus* was terminated by the formation of a basement membrane-like structure on the outermost surface of the cellular capsule. This structure occurred in the early stage of cellular encapsulation and was evident on the exterior surface of the plasmatocyte, when the active haemocytes were attaching to the already melanized microfilariae. The termination structure appears to be laid down by releasing the vesicle inclusions of haemocytes and has similarities in ultrastructure and cationic colloidal gold staining properties with that of mosquito basement membranes.

Key words: *Anopheles quadrimaculatus*, basement membrane-like structure, *Brugia pahangi*, cationic colloidal gold, cellular encapsulation, termination.

INTRODUCTION

Encapsulation is an effective defence response by insects against foreign agents which are too large to be phagocytosed. In most insects, this response involves the accumulation of blood cells (haemocytes) to form a multicellular capsule around foreign agents with or without concurrent melanin deposition. However, in some dipterous insects with few circulating haemocytes, the foreign agents are encased in a humoral melanotic capsule without apparent participation of cells (Gotz, 1986). In addition, the combination of humoral melanization and cellular encapsulation has also been reported in certain mosquito–microfilariae (mf) systems. The mf are first enclosed in an acellular layer of melanin, followed by cellular encapsulation by plasmatocytes, a kind of mosquito haemocyte (Chen & Laurence, 1985; Chen, 1988; Kobayashi *et al*. 1986). However, the cellular layer which enclosed the melanized mf in mosquitoes consisted only of a single layer of cells, whilst other described cellular capsules are comprised of several layers of cells, i.e. more than 20 layers in *Galleria* (Schmit & Ratcliffe, 1977), *Blattella* (Han & Gupta, 1989) and *Melolontha* (Brehelin *et al*. 1975), 15–20 layers in *Periplaneta* (Lackie, Takle & Tetley, 1985), 5–10 layers in *Diabrotica* (Poinar, Leutenegger & Gotz, 1968) and 2–5 layers in *Schistocerca* (Lackie *et al*. 1985).

This variance may be due to the fact that mosquitoes possess fewer haemocytes than do other non-dipterous insects. Consequently, the termination mechanism that prevents additional haemocyte involvement in cellular encapsulation is of prime importance for mosquitoes. It has been suggested that this termination of cellular encapsulation is due to the production of a double membrane-like structure that functions as a boundary to isolate the melanized parasites (Forton, Christensen & Sutherland, 1985; Christensen & Forton, 1986; Chen, 1988). However, the nature of this double membrane-like structure remains unknown. In this study, we demonstrated that the double membrane-like structures that terminated cellular encapsulation of *Brugia pahangi* mf in *Anopheles quadrimaculatus* mosquito was a basement membrane-like structure produced by plasmatocytes.

MATERIALS AND METHODS

Biological materials

The source and maintenance of mosquito *Anopheles quadrimaculatus* and filarial parasite *Brugia pahangi* were essentially the same as that described previously (Chen, 1988). Five to 7-day-old female mosquitoes were fed on rats parasitized by *B. pahangi*

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(2·5–4·2 mf/μl peripheral blood). Engorged mosquitoes were kept at 25±1 °C, relative humidity 70–80 %, and killed at the following time-intervals after feeding: 6, 12, 18, 24 h, 2, 3, 4, 5, 6 or 7 days. The mosquitoes were cold anaesthetized and the encapsulated mf were dissected out in chilled fixatives (3 % glutaraldehyde in 0·1 M cacodylate buffer, pH 7·4, for transmission electron microscopy; 2 % paraformaldehyde and 0·5 % glutaraldehyde in 0·1 M phosphate-buffered saline (PBS), pH 7·3, for cationic colloidal gold labelling).

Transmission electron microscopy

The encapsulated mf were fixed in 3 % glutaraldehyde (Electron Microscopy Science, USA) in 0·1 M cacodylate buffer (Merck, Germany), pH 7·4, for 3 h at 4 °C. Post-fixation was for 1 h at 4 °C in 1 % osmium tetroxide (Electron Microscopy Science, USA) in the same buffer. The fixed specimens were dehydrated in a graded ethanol series (30–100 %) and embedded in Araldite. Ultrathin sections were cut with an ultramicrotome (Reichert Ultracut S, Austria) and were doubly stained in 2 % uranyl acetate (Merck, Germany) for 30 min and 1 % lead citrate (Merck, Germany) for 12 min. Sections were examined and photographed at 100 kV in an electron microscope (JOEL 2000 EX II, Japan).

Cationic colloidal gold labelling

The cationic colloidal gold labelling of encapsulated mf was carried out as described by Juang & Carlson (1994) with some modifications. Briefly, encapsulated mf were fixed in 2 % paraformaldehyde and 0·5 % glutaraldehyde in 0·1 M PBS, pH 7·3, at 4 °C for 30 min. The fixed specimens were washed in 0·1 M NH_4Cl in 0·1 M PBS at 4 °C for 60 min, partially dehydrated in a graded ethanol series (30–70 %) and embedded in LR White resin (Spi Supplies, USA) for 60 h at 45 °C. Ultrathin sections were cut and mounted on Formvar-coated nickel grids. Then the sections were treated sequentially at room temperature on drops of the following: PBS for 10 min; 1 % bovine serum albumin (Boseral, Holland) for 1 h; 10 nm poly-L-lysine-coated colloidal gold particles (Bio Cell, UK, 1:80 dilution in 0·1 M PBS, pH 5·0) for 45 min; deionized water rinse 4×1 min; 2 % uranyl acetate for 30 min; 1 % lead citrate for 12 min. Control sections were treated as described above except that the sections were blocked with 1 mg poly-L-lysine (Sigma, USA, $M_r > 300000$)/ml PBS for 1 h before incubation with poly-L-lysine-coated gold particles.

RESULTS

Formation of basement membrane-like structure

As described previously, the encapsulation of mf in the haemocoels of *A. quadrimaculatus* involves a combination of melanization and cellular encapsulation, the mf were melanized first, followed by a cellular encapsulation by plasmatocytes (Chen & Laurence, 1985). At 12 h post-infection, the formation of a basement membrane-like structure was first evident as the plasmatocytes began attaching to the surface of the already melanized mf and extended to enclose them. The basement membrane-like structure was not seen in areas where plasmatocytes attached to the melanotic mf but observed only on the exterior side of the cell opposite the attaching areas. Fig. 1 shows that the basement membrane-like structure is continually laid down only on those areas of the exterior surface of the plasmatocytes which are spreading over the melanized mf, whilst the rest of the exterior surface is still devoid of deposition. The newly formed basement membrane-like structure is a thin amorphous layer and measures 30–75 nm in thickness. At this time, many vesicles accumulated in the cytoplasm of the plasmatocytes (Fig. 2A), some of them containing inclusions similar to basement membrane-like structure. Fig. 2B shows small vesicles that appear to be in the process of bursting, thus releasing their inclusions onto the exterior surface of the plasmatocyte. The deposition of basement membrane-like structure was not seen in the spaces between plasmatocytes (Fig. 3A). At 48 h post-infection, the outer surface of the completed capsule, which consisted of an inner melanin layer and an outer cellular layer, was totally enclosed with a basement membrane-like structure (Fig. 3B). At this time, the basement membrane-like structure still retained its amorphous appearance and became slightly condensed (25–40 nm thick) as compared to that of the newly formed structure. The ultrastructural appearance and thickness of the completed basement membrane-like structure was similar to that of the basement membrane of mosquito fat body tissues (15–35 nm thick) (Fig. 3C).

Once the formation of basement membrane-like structure was completed, no further recruitment of plasmatocytes to the melanized mf occurred. Occasionally, however, a few plasmatocytes were seen attaching to the outer surface of the original cellular layer. These cells were elongated and heavily pigmented, but did not penetrate through the basement membrane-like structure. The deposition of basement-membrane like structures resulted in the formation of a boundary that completely enclosed the cellular capsule of melanized mf, and separated them from haemolymph.

Cationic colloidal gold labelling

Both the basement membrane-like structure, which was laid down on the exterior surface of plasmatocytes enclosing the melanized mf (Fig. 4A, B), and the basement membrane of fat body tissues (Fig.

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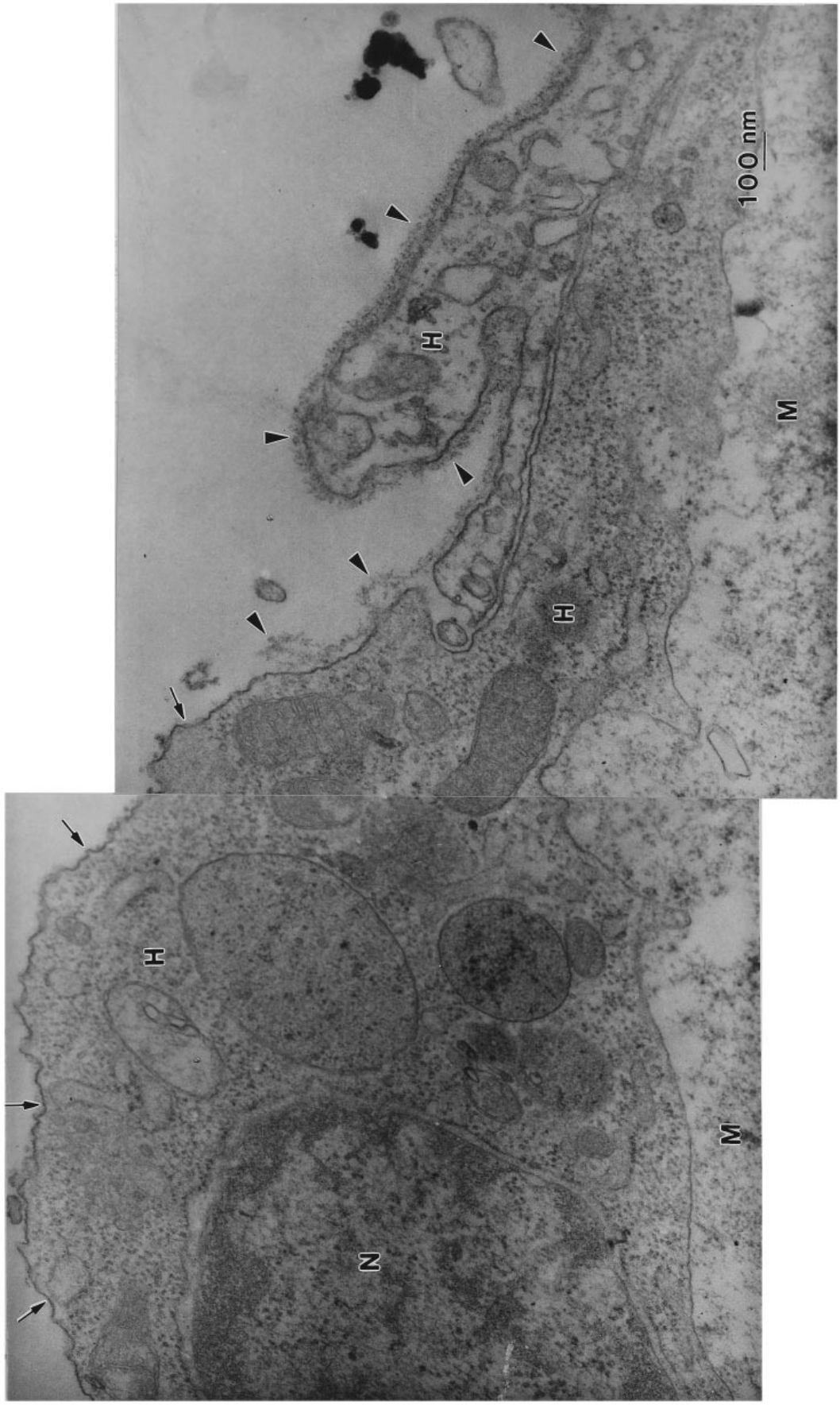

Fig. 1. Basement membrane-like structure (arrowheads) is laying down on the surface of plasmatocyte (H) which is spreading over a melanotic capsule (M), 12 h post-infection. Note that the deposition of basement membrane-like structure can be seen only on some areas of the plasmatocyte, whilst the rest of the surface (small arrows) are still devoid of deposition. N, nucleus.

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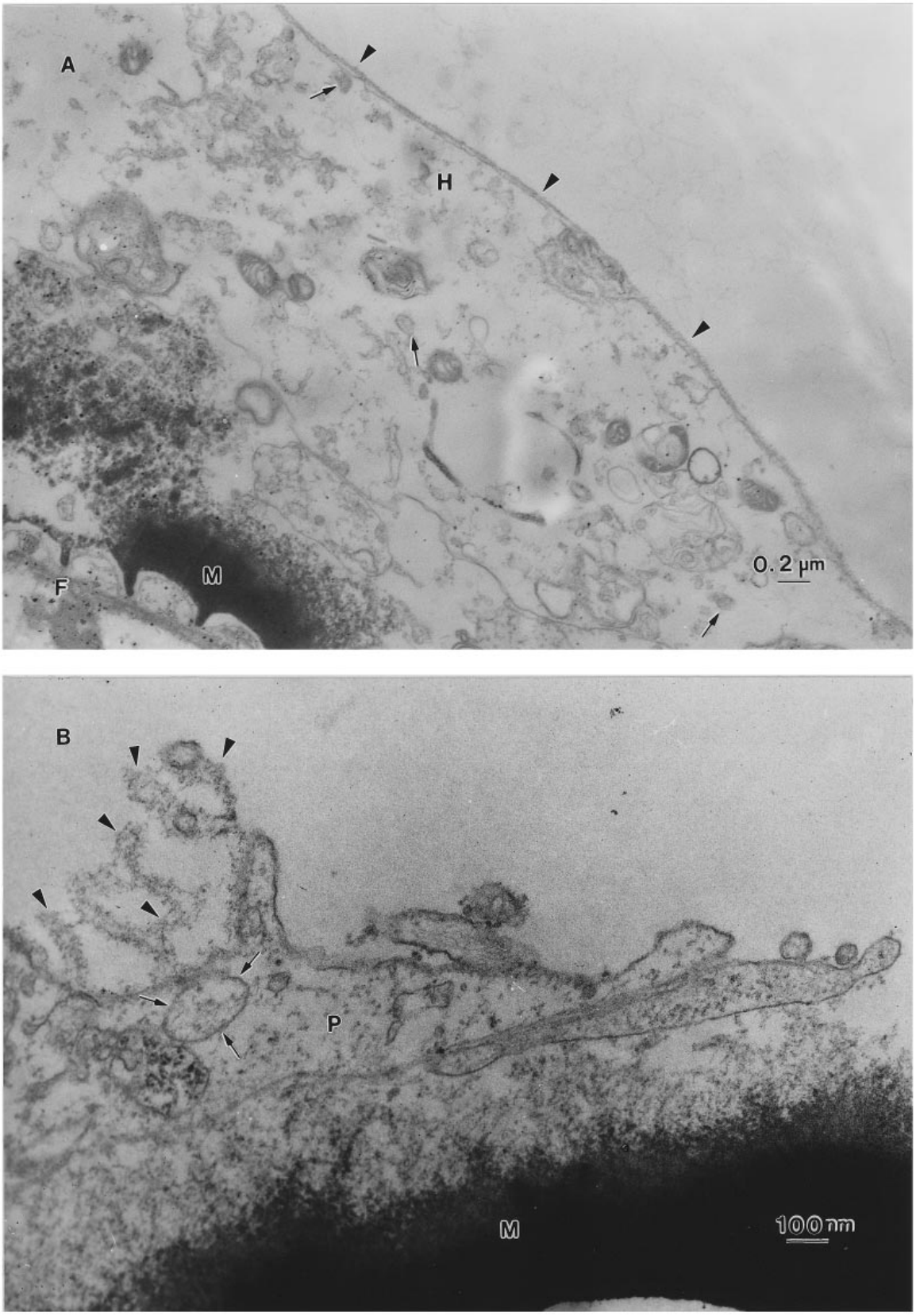

Fig. 2. (A) Abundant vesicles (small arrows) of various sizes are present in the cytoplasm of a plasmatocyte (H) which encloses a melanized microfilaria (F), 12 h post-infection. Arrowheads indicate the basement membrane-like structure. M, melanotic capsule. (B) Pseudopodial extension (P) of a plasmatocyte extending to enclose a melanotic capsule (M), 12 h post-infection. Note a small vesicle (arrows) apparently releasing its basement membrane-like structure (arrowheads) on the surface of the pseudopodial extensions.

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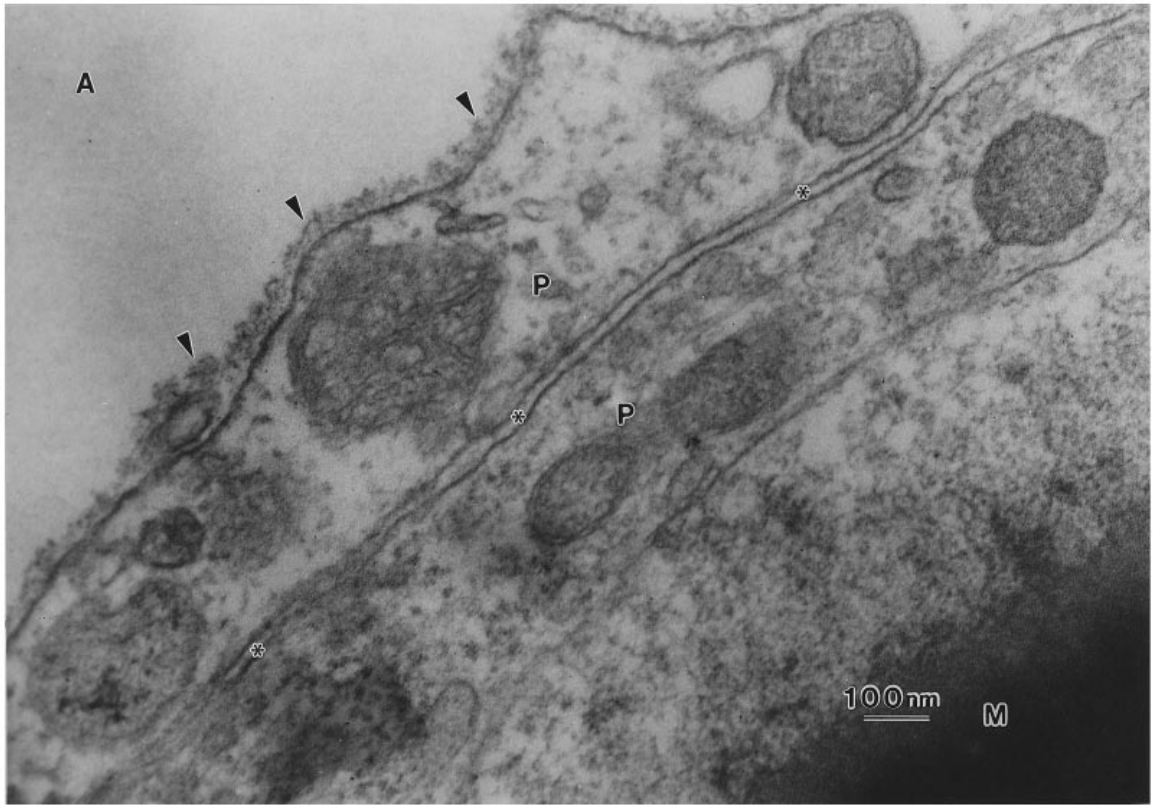

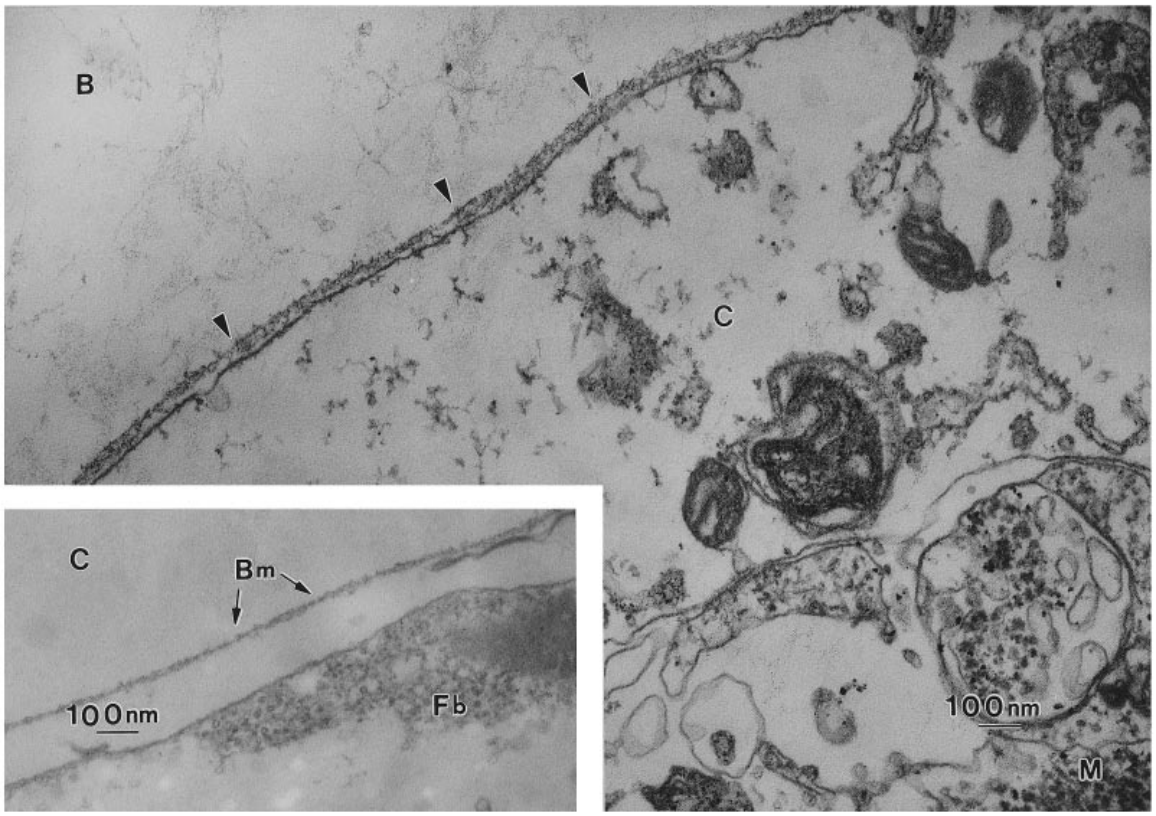

Fig. 3. (A) Two pseudopodial extensions (P) spread over the surface of a melanotic capsule (M), 24 h post-infection. Note that 1 pseudopodial extension has spread over the other and the basement membrane-like structure (arrowheads) can only be seen on the outermost surface of pseudopodial extension. No basement membrane-like structure is laid down in the space between the 2 pseudopodial extensions (*). (B) The outer surface of the cellular capsule (C) is completely enclosed by basement membrane-like structures (arrowheads), 48 h post-infection. M, melanotic capsule material. (C) Basement membrane (Bm) of fat body (Fb).

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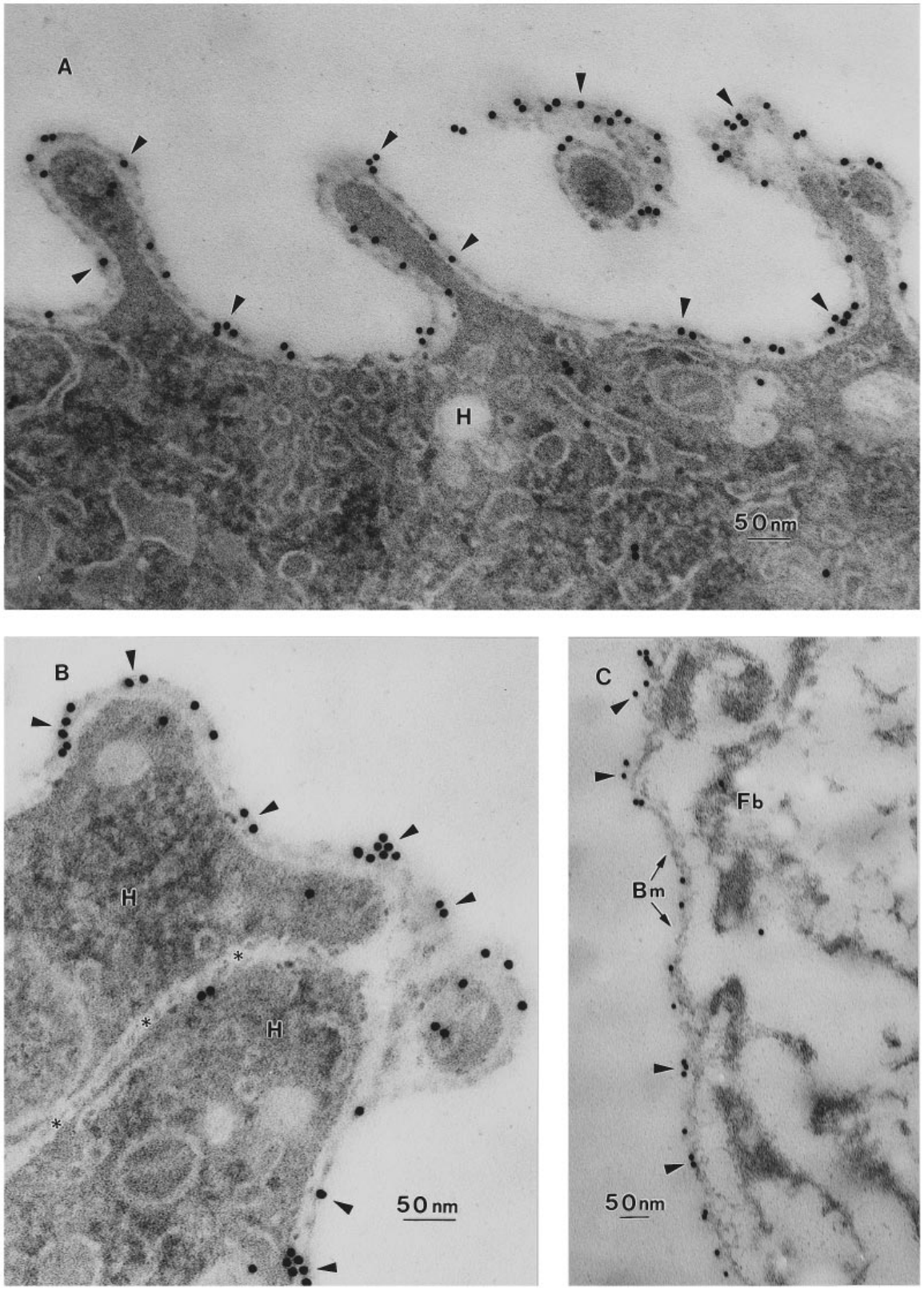

Fig. 4. (A) Cationic colloidal gold labelling on the plasmatocyte (H) which is enclosing a melanized microfilaria, 9 days post-infection. Note that gold particles label intensely on the basement membrane-like structure (arrowheads) but only a few gold particles are present on the cytoplasm of the haemocyte. (B) Cationic colloidal gold labelling on 2 plasmatocytes (H) which are enclosing a melanotic capsule, 9 days after infection. Note that 2 haemocytes interlock with each other. Gold particles label intensely on the basement membrane-like structures (arrowheads) but, very few gold particles are present on the cytoplasm and the spaces (*) between the 2 plasmatocytes. (C) Cationic colloidal gold labelling on the basement membrane (Bm) of fat body (Fb). Note gold particles label intensely on the basement membrane (arrowheads).

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4C), were heavily labelled by cationic colloidal gold particles. No significant labelling was seen on the cell membrane and cytoplasm of the plasmatocytes, and none of the spaces between haemocytes was labelled. Negative control sections treated with poly-L-lysine showed no significant gold labelling of any of the basement membrane-like structure or the plasmatocytes (data not shown).

DISCUSSION

Despite the fact that encapsulation is a commonly observed defence reaction of insects against parasites, little is known about the termination mechanism of this reaction. Lackie *et al*. (1985) showed that the surface of old capsules from the cockroach, *Periplaneta americana*, is covered by sheets of homogenous granules. The coating sheet appears ultrastructurally similar to the subepidermal basement membrane, and both of these layers stain with Alcian blue. How this acellular coating sheet forms is unknown, but once present, attachment of additional haemocytes to the capsule ceased. Similar acellular coating sheets were also found in the cockroach *Blattella germanica* (Han & Gupta, 1989). Forton *et al*. (1985) demonstrated that a double membrane-like structure functioned as a boundary to isolate the melanized mf which prevented additional haemocyte involvement in encapsulation in the mosquito *Aedes trivittatus*. This double membrane-like structure appeared as a single membrane initially then, later, the second membrane formed inside of the initial membrane. However, they found that the complexity of this double-membrane formation at times made interpretations difficult. More recently, Pech & Strand (1996) developed an *in vitro* encapsulation system by culturing haemocytes from *Pseudoplusia includens* and found that termination of *in vitro* capsule formation occurred when a subpopulation of granular cells formed a monolayer around the periphery of the capsule. However, they did not describe how the granular cells terminated the cellular encapsulation *in vitro*.

The results of the present study demonstrate that the outermost surface of cellular capsules which encase the melanized *B. pahangi* mf in the haemocoels of *A. quadrimaculatus* was completely enclosed by a basement membrane-like structure. The basement membrane-like structure has similarities in ultrastructure and cationic colloidal gold binding properties with those of mosquito basement membrane and was apparently laid down by the release of vesicle inclusions of plasmatocytes. In the literature, the participation of haemocytes in basement membrane formation in insects is a long-standing controversy. Although Wigglesworth has published a series of papers demonstrating that *Rhodnius* haemocytes spread over the basement membranes and discharged their inclusions to become incorporated into the membrane (Wigglesworth, 1956, 1973, 1979). Ashhurst (1979), however, regarded the involvement of haemocytes in the secretion of connective tissues as circumstantial. Nevertheless, studies using monoclonal antibodies have confirmed that insect haemocytes contributed to basement membrane formations in embryonic locusts (Ball *et al*. 1987), and in developing wings of *Manduca sexta* (Nardi & Miklasz, 1989). Chain, Leyshon-Sorland & Siva-Jothy (1992) described 3 monoclonal antibodies generated against haemocytes of *P. americana* which cross-reacted with the basement membrane lining the haemocoel and the periphery of capsules. Sass, Kiss & Locke (1994) further demonstrated that cuticle peptides occurred both in haemocyte secretary vesicles and the outer layers of the basal lamina in the caterpillar *Calpodes ethlius*, and strongly suggested that some connective tissue components are laid down directly by haemocytes as they move over basal lamina surfaces, as Wigglesworth suggested almost 40 years ago. Consequently, it would be appropriate to suggest that the double membrane-like structure formed at the outermost cellular capsules of the melanized mf in the haemocoels of mosquitoes is a basement membrane-like structure. The basement membrane-like structure was laid down when the active haemocytes were attaching to the already melanized mf. It occurred in the very early stages of the cellular encapsulation, which seemed to prevent further attachment of any additional haemocytes at this point, leaving only 1 layer of cells, which encased the melanized mf. In conclusion, the cellular encapsulation of *B. pahangi* mf in the haemocoel of *A. quadrimaculatus* was terminated by the formation of basement membrane-like structure on the surface of the cellular capsule.

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