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Presence of peritrophic-like membranes in the intestine of three bacteriophagous nematodes (Nematoda : Rhabditida)

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Summary – Ultrastructural analysis shows the presence of membranes originating at the top of the intestinal microvilli, along the entire length of the intestine in the three rhabditid nematodes *Caenorhabditis elegans, Panagrolaimus superbus* and *Acrobeloides maximus*. The membranes allow the passage of fluorescein isothiocyanate, methyl red, neutral red and acridine orange, but allow only sparse passage of ferritin molecules. Upon the introduction of a sublethal dose of sodium azide, the intestinal lumen displays an increased secretion of membrane layers. Whole mount staining of the nematodes with the *Solanum tuberosum* and *Triticum vulgare* lectins, known to bind with high affinity to chitin, shows only specific binding of *Solanum tuberosum* lectin to the brush border in all developmental stages of *C. elegans, P. superbus* and *A. maximus*. The results reveal the presence of membranes in the intestine of three species of one of the most ancient metazoan phyla, exhibiting morphological and functional characteristics reminiscent of peritrophic membranes in insects.

Résumé – ***Présence d'une membrane pseudo-péritrophique dans l'intestin de trois nématodes bactériophages (Nematoda : Rhabditida)*** – Chez trois nématodes Rhabdities, *Caenorhabditis elegans, Panagrolaimus superbus* et *Acrobeloides maximus,* les analyses ultrastructurales ont démontré la présence d'une membrane prenant naissance à l'extrémité des microvillosités intestinales, et ce sur l'entière longueur de l'intestin. Ces membranes permettent le passage de l'isothiocyanate de fluorescéine, du rouge de méthyle, du rouge neutre et de l'orange d'acridine, mais un passage très limité des molécules de ferritine. Après introduction d'une dose subléthale d'azide de sodium, la lumière intestinale est le siège d'une augmentation de la sécrétion des couches de la membrane. Des colorations *in toto* des nématodes avec des lectines provenant de *Solanum tuberosum* et *Triticum vulgare,* connues pour leur grande affinité avec la chitine, ont seulement montré une liaison spécifique de la première avec la frange en brosse, et ce chez tous les stades des trois nématodes considérés. Ces résultats révèlent dans l'intestin de trois espèces appartenant à un des plus anciens phylums de métazoaire, la présence de membranes possédant des caractéristiques morphologiques et fonctionnelles rappelant les membranes péritrophiques des insectes.

Key-words : peritrophic membranes, Rhabditida, TEM, lectins.

Peritrophic membranes are best known from the intestine of insects where they exist in a wide variety of shapes and sizes, active in protective, digestive, defensive and sometimes cocoon forming functions (Peters, 1992). During ultrastructural analysis of the intestine of free-living bacteriophagous nematodes, several membrane layers were identified either on top of the microvilli or secreted into the intestinal lumen (Borgonie *et al.*, 1994*a*). Since these membranes were highly reminiscent of insect peritrophic membranes, several of the characteristics of peritrophic membranes were evaluated in three bacteriophagous nematodes.

Material and methods

NEMATODES

Three free-living nematode species belonging to the order Rhabditida : *Caenorhabditis elegans* var. *Bristol* (Rhabditidae), *Panagrolaimus superbus* (Panagrolaimidae) and *Acrobeloides maximus* (Cephalobidae).

MONOXENIC AND AXENIC CULTURE

All cultures were sterilised using standard hypochlorite solution according to Sulston and Hodgkin (1988). Nematodes were cultured on *Escherichia coli* and generally handled according to Brenner (1974). Stock cultures were kept at 20 °C. Nematodes were cultured axenically according to Vanfleteren *et al.* (1990). Briefly, a mixture of 3 % soy peptone and 3 % yeast extract was autoclaved under standard conditions. 0.1 % haemoglobin solution was added aseptically afterwards. The hemoglobin solution was made by dissolving 5 g haemoglobin (Serva) in 100 ml 0.1 M KOH and autoclaved for no longer than 10 min.

A. maximus could not be cultured indefinitely on this medium without amending the basic sterile medium. An additional 250 µl haemoglobin solution/100 ml sup-

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ported indefinite culture. No suitable axenic medium has been identified that allows an indefinite culture of *P. superbus*. On the basic axenic medium three to four generations can be supported. Addition of more haemoglobin or heated liver extract showed no improvement.

Transmission electron microscopy (TEM)

i) Embedding in Spurr was according to Van De Velde and Coomans (1989). Young females were collected in distilled water and fixed at room temperature in Karnovsky fixative (2 % paraformaldehyde, 1 % glutaraldehyde and 2.5 % acrolein in 0.2 M Na-cacodylate buffer pH 7.2). After 30 min nematodes were transferred to Karnovsky minus acrolein and nematodes were cut in half, transferred again to Karnovsky with acrolein and left overnight at 4 °C. Nematodes were rinsed in 0.2 M Na-cacodylate buffer pH 7.2 for 8 h at room temperature and then postfixed for 48 hours in 2 % OsO_4 in 0.2 M Na-cacodylate buffer pH 7.2 at 4 °C. Nematodes were transferred to 50 % alcohol, rinsed several times until the black colour disappeared and were subsequently transferred to 2 % uranyl acetate in 50 % alcohol for 1 h. Dehydration was performed using 70, 90 and 100 % alcohol three times 20 min each at room temperature. Nematodes were put into 100 % alcohol/Spurr (1:1) overnight, absolute alcohol/Spurr (1:2) for 8 h and transferred to 100 % Spurr overnight at 4 °C. Polymerization was carried at 70 °C for 12 h. Alternatively, Epon was used as embedding medium.

ii) LR White fixation was performed according to a protocol kindly provided by N. Kershaw (MRC-LMB, UK). Nematodes were collected in 0.1 M HEPES (pH 7.5), put on ice and fixed in freshly prepared 4 % paraformaldehyde in 0.1 M HEPES (pH 7.0). Nematodes were cut in the fixative and left on ice for 30 min. Nematodes were subsequently washed twice in 0.1 M HEPES (pH 7.0). Nematode pieces were transferred to small agar blocks to facilitate processing. Dehydration was performed twice in 15 % ethanol (15 min each) once in 30 % alcohol (15 min), once in 50 % alcohol (15 min) and twice in 70 % alcohol (30 min). Nematode samples are transferred into 2:1 LR White/70 % ethanol for 30 min and transferred to 100 % LR White overnight. The resin was changed several times before the nematodes were put in airtight capsules for polymerization at 50 °C for 24 h.

80 nm thick sections were made using a Reichert OMU-2 ultramicrotome. Formvar coated single slot copper grids were used. Sections were poststained with an LKB ultrastainer for 30 min in uranyl acetate at 40 °C and 5 min in lead stain at 20 °C. Electron microscopy was performed using a Siemens Elmiskop 1A, operating at 80 kV.

Whole mount nematode lectin staining

A detailed analysis of optimal fixation and treatment of nematode tissue for lectin histochemistry has been described previously (Borgonie *et al.*, 1994*b*). Briefly, slides were coated with 0.1 % poly-L-lysine (MW > 320.000, Sigma) and a drop of nematodes in distilled water (mixed culture containing all developmental stages, approximately 200 animals) was put on the slide and covered with a coverslip. Excess water was removed and the slide was dipped in liquid nitrogen for 2 min. The coverslip was pried off and the slide immersed in pre-cooled (– 20 °C) acetone for 5 min. Rehydration was done through a 20 % acetone series. Incubation was performed overnight at 4 °C with 50 µl lectin from a 100 µg/ml *Solanum tuberosum* lectin (STA) of *Triticum vulgare* lectin (WGA; both from Sigma) stock solution in phosphate buffered saline (PBS; 50 mM Na_2HPO_4, 140 mM NaCl, pH 7.2).

Controls

Controls were carried out by preincubating the FITC (fluorescein isothiocyanate) – labelled STA with 0.03 M of N, N′, N″ – triacetyl chitotriose (Sigma). Lectin and sugar were mixed for 1 hour at room temperature before the mixture was added to the slides. A second control testing binding specificity was done by oxidizing sugar residues in the nematode tissue. This was achieved by preincubating the nematodes overnight in the dark in 50 mM $NaIO_4$ in 100 mM potassium acetate buffer (pH 4.5) and subsequent washing three times in cold PBS (4 °C) prior to lectin incubation. All observations were made 30 min after the slides were sealed. Observations were made using a Leitz microscrope equipped for fluorescence microscopy. Photographs were taken on Kodak Ektachrome 160T film and processed commercially.

Chitinase and β-N-acetylglucosaminidase tissue digestions

To test the possible presence of chitin in the intestinal membrane structures, the nematode tissue was first enzymatically treated in a two step reaction to remove chitin before staining with STA. Nematodes were freeze-cracked, rehydrated in acetone only as described above, and subsequently incubated in 16 mg/ml (10-30 units/mg) *Serratia marcescens* chitinase (Sigma) in 50 mM NaCl, 70 mM KCl, 2.5 mM $MgCl_2$ and 2.5 mM $CaCl_2$, pH 6.0 for 1, 6 and 12 h at 25 °C. The slides were washed three times for 15 min in 50 mM sodium citrate buffer (pH 4.0) and for the second step the tissue was incubated with 0.5 mg/ml (30 units/mg) *Aspergillus niger*-β-N-acetylglucosaminidase (Sigma) in 50 mM sodium citrate buffer, pH 4.0 for 1, 6 and 12 h at 25 °C. After washing the slide three times for 5 min each the nematodes were incubated with STA overnight as described above. The following incubation combinations were performed : chitinase only, chitinase and β-N-acetylglucosaminidase and β-N-acetylglucosaminidase only. To determine the effect of the enzyme buffers only on staining patterns, controls were carried out by

incubating nematodes in the respective enzyme buffers in the same incubation conditions but without enzyme. To test the activity of the enzymes, a small piece of chitin from crab shells suitable for the analysis of chitinase (Sigma C 3641) was incubated with the enzymes as described for the incubation of the nematode tissue. Furthermore it was determined whether the egg shell (containing chitin) still showed lectin binding after the removal of chiting using the enzymes.

ACRIDINE ORANGE, FLUORESCEIN ISOTHIOCYANATE, METHYL RED, NEUTRAL RED, FERRITIN AND NAN_3

Nematodes were washed off the agar plates and rinsed three times in PBS, pH 7.2. Acridine orange (AO), fluorescein isothiocyanate (FITC), methyl red (MR) and neutral red (NR) were dissolved in PBS and sonicated extensively to break up a small amount of dye aggregates that did not solubilize. Insoluble material was removed by centrifugation at 10 000 rpm for 1 min in a microfuge. Incubation was carried using 50 µl of a mixed nematode culture to which 50 µl of dye solution was added. The final concentrations were : 0.001 % for AO, 1 % for FITC, 2.5 % for MR and 1 % for NR (concentrations are approximate since a small amount was lost during centrifugation). Incubation was done at 22 °C for 24 h. After incubation the nematodes were washed five times in PBS. For observations nematodes were mounted on 2 % agarose pads. AO and FITC staining were observed using a microscrope equipped with FITC optics. 10 mg cadmium-free horse spleen ferritin (Serva) was dissolved in 1 ml PBS and sonicated briefly. 250 µl of the ferritin solution was added to 250 µl nematode suspension at 22 °C for 2 h, after which nematodes were processed for TEM using Spurr as embedding medium. NaN_3 was dissolved in PBS and added for 1 hour at 22 °C to a nematode suspension at a final concentration of 0.5 mM. Seemingly fully healthy nematodes were prepared for TEM using Spurr as embedding medium.

Results

Cross-sections made at three levels of the intestine (posterior to the pharynx, at midlevel and posterior intestine) and longitudinal sections of the nematodes *C. elegans, P. superbus* and *A. maximus* showed that at the top of the microvilli, multi-layered, mostly discontinuous threads were secreted into the intestinal lumen (Fig. 1). Different types of membranes were observed. In *A. maximus* the membranes were discontinuous and appeared as a string of pearls (Fig. 1 A). In *C. elegans* two types were identified. The first type appeared as a random network of fine threads in the lumen (Fig. 1 B) and a second type as a discontinuous, double membrane, comparable to the membrane observed in *A. maximus*. In *P. superbus* only one type was observed, being morphologically identical to the second type in *C. elegans*. When the nematodes were cultured on bacteria, the threads were sometimes seen to be swirled around the intestinal lumen contents. In nematodes cultured in sterile axenic medium the threads were situated freely in the lumen. To exclude the possibility that the threads were artefacts induced by fixation, embedding and/or processing for TEM, two different fixation schemes and three embedding media were used. The threads were readily identified whatever processing was used.

Ultrastructure of the intestine after feeding the three nematodes on a sublethal dose of the ATP depleting poison NaN_3 showed massive secretion of long, dark multi-layered stretches of membrane into the intestinal lumen (Fig. 1 C) in amounts never observed in untreated animals. The secreted layers were longer than generally seen in untreated nematodes and did not resemble any of the two types observed in untreated *C. elegans*.

To test a possible barrier function of the membranes, nematodes were fed fluorescein isothiocyanate (FITC), acridine orange (AO), methyl red (MR), neutral red (NR) and cadmium-free horse spleen ferritin. FITC, AO, NR and MR were readily found in the intestinal cells of the nematodes. Ferritin filled the entire lumen of the intestine (Fig. 1 D), individual ferritin particles were only sparsely found between microvilli and in vacuoles in the intestine.

Since insect peritrophic membranes often contain chitin (Peters, 1992), we used FITC labelled STA and WGA lectins that bind strongly to oligomers of β(1-4) N-acetyl-D-glucosamine, present in the polymere chitin, to determine the intestinal staining patterns in whole mount nematodes (Fig. 2). WGA did not stain the brush border or the intestinal lumen in any of the nematode species. Using STA, however, several structures were stained in all three nematode species (Fig. 2). Binding to the brush border was always over the entire length of the intestine (Fig. 2 A, B) in all juvenile and adult stages, but not in embryos ready to hatch. Staining was strong in *P. superbus* and *A. maximus,* but almost imperceptible in *C. elegans*. Staining of the intestinal border was always present and visible as a distinct line (Fig. 2 A). In the lumen the staining was variable and hazy (Fig. 2 A, B). Lectin staining was specific in the three species studied since pre-incubation with N, N′, N′′-triacetyl chitotriose inhibited staining. Staining was also abolished after the nematode carbohydrates were oxidized using $NaIO_4$. Animals cultured either on bacteria or in sterile axenic medium for several generations gave identical results.

Enzymatic digestion of the nematode tissue using either chitinase alone or in a two-step reaction with β-N-acetylglucosaminidase before STA staining revealed no differences between enzymatically untreated (Fig. 2 B) and treated tissue (Fig. 2 C, D). The controls showed that the buffers alone did not affect staining patterns. The digestion of the crab shell chitin showed that the enzymes were active. Additional evidence that the enzymes were active came from the disappearance of

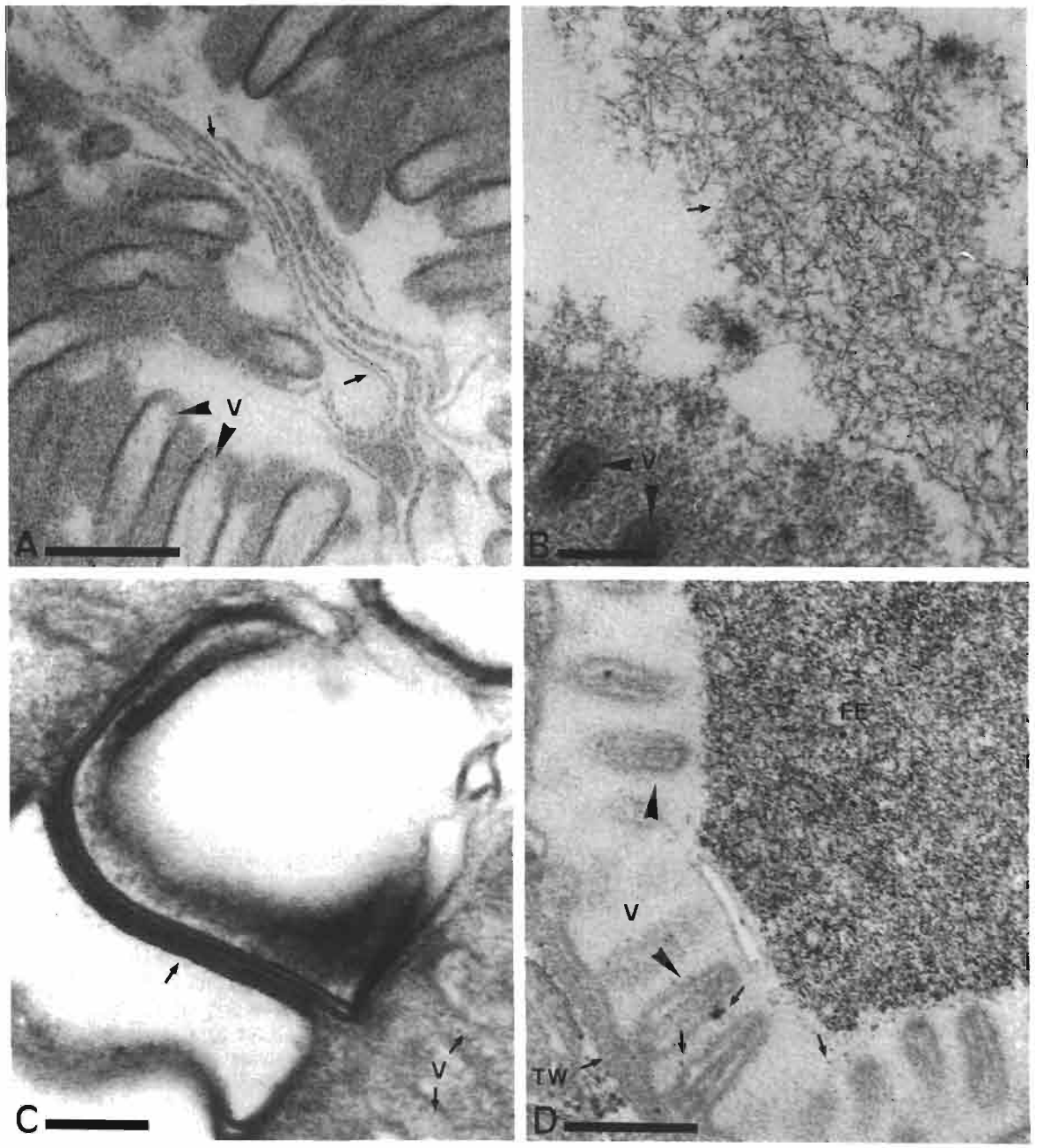

Fig. 1. *TEM of secreted membranes present in intestinal lumen of axenically cultured rhabditid nematodes. A : Double layered threads (arrows) in the intestinal lumen of* A. maximus *embedded in Spurr; B : Irregular meshwork of fine threads (arrow) in the intestinal lumen of* C. elegans *embedded in Epon; C : Massive secretion of membranes (arrow) in the intestine of* C. elegans *embedded in Spurr after feeding in the presence of* NaN_3*; D : Barrier function demonstrated in* C. elegans *embedded in Spurr after feeding in the presence of cadmium-free horse spleen ferritin. Bulk of the ferritin remains in the lumen and does not penetrate the glycocalyx between microvilli (Small arrows indicate presence of small amounts of ferritin between the microvilli.* V : *microvilli,* TW : *Terminal web,* FE : *ferritin.* Bars A, D = 1 μm; B, C = 0.5 μm).

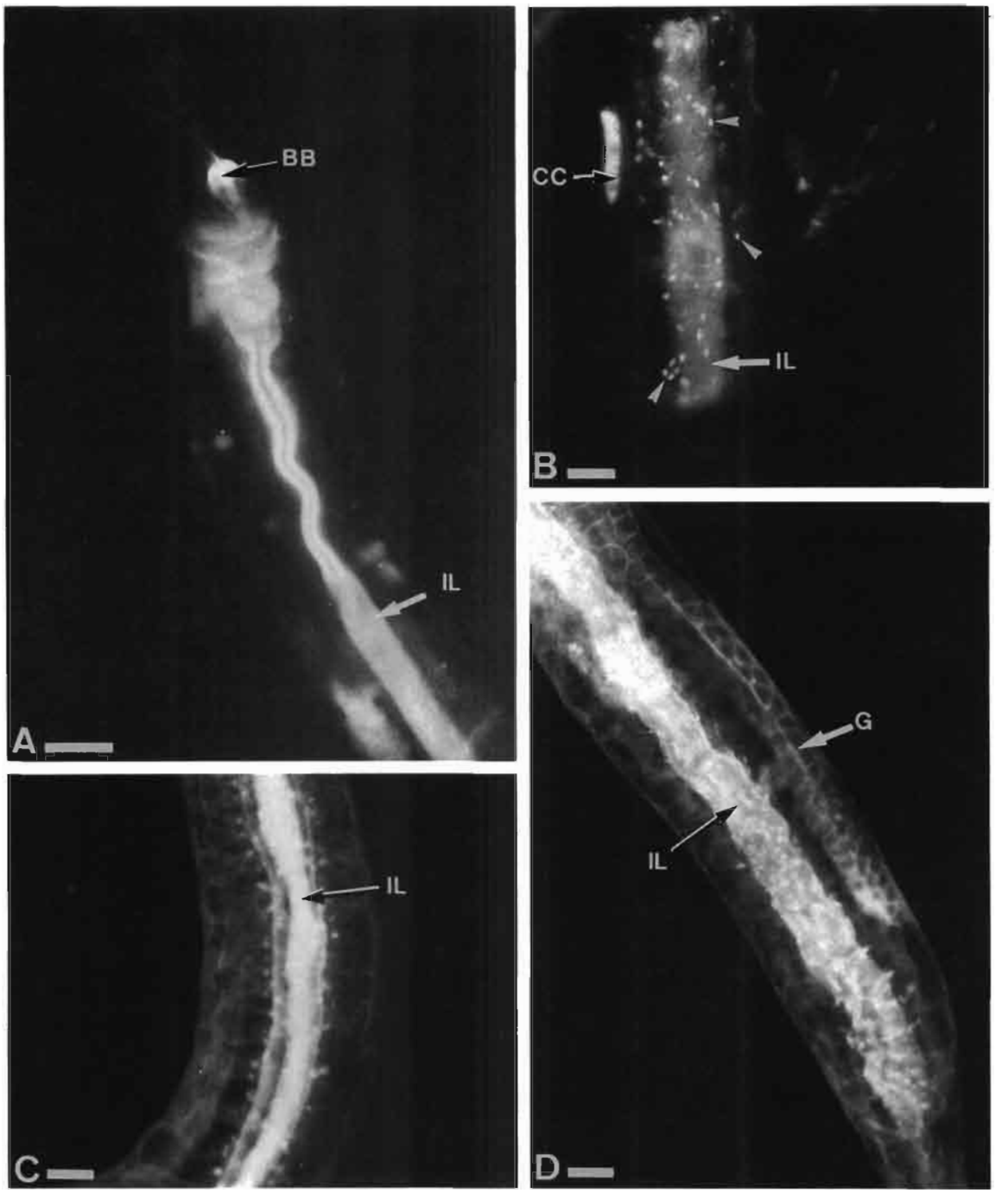

Fig. 2. *Whole mount internal STA staining. A : P. superbus, showing staining of the brush border, while staining of the lumen is variable; B : A. maximus, besides staining of the lumen and brush border, arrowheads point to irregular fluorescent spots in intestinal cells. Untreated tissue; C : A. maximus, after the nematode tissue had been incubated with chitinase for 12 h. No difference in staining with untreated nematodes was visible; D :* A. maximus *after the nematodes tissue had been incubated with chitinase and β-N-acetylglucosaminidase. No difference with untreated tissue evident (*BB : *basal bulb,* IL : *intestinal lumen,* CC : *coelomocyte,* G : *gonad. Anterior top, adult females.* Bars : A = 25 μm; B, C, D = 20 μm).

staining of the eggshell after enzyme treatment. This was expected since the nematode eggshell is known to contain chitin.

Discussion

Since the membranes were observed in three different rhabditid species, using two fixatives and three embedding media, the possibility that they are an artefact as a consequence of processing for TEM can be dismissed. Similarly, the observation of the membranes in nematodes cultured on sterile axenic media for several generations excludes the possibility that the membranes derive from assorted bacterial debris.

TEM of nematodes fed on ferritin showed that the layer immediately on top of the microvilli acts as a barrier to the bulk of the ferritin molecules allowing but sparse passage of molecules at any given time. Clokey and Jacobson (1986) fed several fluorescein-labelled markers to *C. elegans* and reported that the fluorescein-labelled lectin concanavalin A and latex spheres (0.25 μm) were not internalized in the intestinal cells of *C. elegans*. However, these components may simply not trigger the internalization process (e.g. absence of the proper receptor). In contrast ferritin was shown to be a suitable nutrient for culturing *C. elegans* in chemically defined media (Vanfleteren, 1980).

Massive secretion of membranes that envelop and neutralize poisons and pathogens is a widely claimed defensive function for peritrophic membranes in insects (Peters, 1992). Accordingly massive secretion of membranes was observed after nematodes were fed with sodium azide.

The variable and hazy staining of the lectins in the intestinal lumen suggests that at least part of the carbohydrate bound by the lectin was secreted into the lumen. In insects peritrophic membranes are secreted during feeding, rarely embryonically, indicating that secretion is linked to feeding (Peters, 1992). Accordingly we found that the STA staining was absent in embryos ready to hatch, whereas hatched first stage feeding juveniles exhibited strong staining. Staining patterns were identical irrespective of whether nematodes were cultured on bacteria or on sterile axenic medium, indicating that the lumen content did not contribute to the staining pattern observed. However, the absence of staining with WGA lectin weakens the possibility that the intestinal staining pattern is due to chitin. WGA binds strongly with the chitobiose core of asparagine linked oligosaccharides, specifically with the Manβ (1,4) GlcNAcβ (1,4) GlcNAc trisaccharide (Anon., 1992). Although it has been reported that other lectins sharing similar carbohydrate binding properties as WGA do not necessarily exhibit similar staining patterns (Anon., 1992), the negative results with the enzymatic treatment of the tissues convincingly indicates that the binding of STA is not due to the presence of chitin. Nevertheless the difference in staining intensity between *C. elegans* and the other two species does indicate biochemical differences of the intestinal secretions between the three species.

Considering the functions proposed for peritrophic membranes, it is likely that they will predominantly be found in free-living nematodes as they ingest huge amounts of bacteria and debris. In free-living marine nematodes processed for TEM, membranes at the top of the microvilli have been described, although no biochemical or functional data were presented (Deutsch, 1978; Nuss, 1985; Van De Velde & Coomans, 1989). Specialized feeding regimes may explain the absence of peritrophic membranes in the animal parasitic nematodes *Ascaris suum* and *Leidynema appendiculata* as reported by Peters (1992). For the same reason we doubt the presence of peritrophic membranes in the more advanced plant-parasitic nematodes. These nematodes no longer have microvilli and the nematodes probably feed on predigested plant fluids (for review see Geraert, 1992).

Peritrophic membranes have also been observed in Priapulida and Sipunculida (Peters, 1992). The precise affiliation of these two groups with other lower metazoan taxa is still a matter of debate, but they are generally placed near the pseudocoelomatic group which includes the nematode phylum. We believe that peritrophic membranes in the nematode, priapulid and sipunculid intestine are archaic acquisitions in the oldest phyla in a line leading to the insects (Vanfleteren *et al.*, 1990). We anticipate that the occurrence of peritrophic membranes will prove to be more generally distributed among lower metazoan phyla.

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