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Lysosomal and pseudocoelom routing protects *Caenorhabditis elegans* from ricin toxicity

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Summary – The resistance of the nematode *Caenorhabditis elegans* towards the highly potent toxin ricin has been studied. Incubation of *C. elegans* in ricin did not affect life span or progeny production. However, micro-injection of the ricin A-chain into the distal, syncitial gonad caused degeneration and sterility in test specimens, confirming that *C. elegans* ribosomes are sensitive. Using transmission electron microscopy, it was observed that ricin is effectively internalised into the intestinal cells. When pre-labelled with gold, the toxin reached only the lysosomes. When native toxin was used, the toxin was either routed to the lysosomes or underwent transcytosis to the pseudocoelomatic cavity and incorporation into embryos. None of the ricin reached either the trans Golgi network or the Golgi apparatus, considered essential for toxicity. The observed oral non-toxicity is therefore due to alternate sorting of the toxin, a mechanism not previously observed. The data indicate that, although ricin can opportunistically bind to, and be internalised by, cell surface receptors, these receptors are not sufficient to elicit toxicity.

Keywords – acridine orange, electron microscopy, endocytosis, intestine, resistance.

Ricin is a representative of a group of highly toxic plant and bacterial proteins generally named ribosome inhibiting proteins (RIP). Ricin is isolated from the seeds of castor beans (*Ricinus communis*) and consists of two polypeptide chains, A (32 kDa) and B (36 kDa), linked by a single disulphide bridge. The A-chain is an *N*-glycosidase that inactivates ribosomes by specifically removing a single adenine residue from 28S ribosomal RNA, thereby inhibiting protein synthesis (Endo & Tsurugi, 1987), whereas the B-chain is a galactose-specific lectin that can bind to mannose receptors or terminal galactosyl-residues in a variety of glycoproteins and glycolipids on cell surfaces. Upon binding, ricin is internalised by endocytosis. Ricin is then transported to lysosomes and the trans Golgi network. After reaching the rough endoplasmic reticulum, the A-chain translocates to the cytoplasm either from the rough endoplasmic reticulum or from an as yet unknown intracellular compartment into the cytoplasm where it exerts its toxic effect (Lord *et al.*, 1994; Sandvig & Van Deurs, 1994).

When nematodes were used to study internal staining patterns using lectins, strong, specific staining with ricin was observed of the intestinal brush border in two of the three nematode species used, including *Caenorhabditis elegans* (Borgonie *et al.*, 1994). Surprisingly, when *C. elegans* was incubated with high concentrations of ricin, no toxic effect was observed, despite the presence of the highly conserved ricin A-chain target sequence in *C. elegans* rRNA (Wilson *et al.*, 1994).

In the present study, we analysed the ricin resistance by incubating the nematode *C. elegans* in ricin and following the pathway of the toxin as it enters the intestine and beyond.

Material and methods

NEMATODES

Caenorhabditis elegans N2 was sterilised for monoxenic culture according to Sulston and Hodgkin (1988). Nematodes were cultured on *Escherichia coli* and generally handled according to Brenner (1974).

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Toxicity testing

Because the commercial ricin preparation offered by Sigma (St Louis, MO, USA) contains the preservative NaN_3, all ricin samples were extensively dialysed. Typically, 100 μl of ricin preparation was dialysed overnight against 4 l of PBS (50 mM NaH_2PO_4, 140 mM NaCl, pH 7.2) at 4°C. For toxicity testing approximately 200 nematodes were collected in PBS. The PBS was aerated extensively to promote digestion of remaining bacteria. Nematodes were incubated in a Falcon 48-well microtiter plate with 100 μl of the dialysed ricin solution. Final ricin concentrations tested ranged from 10-50 μg/100 μl.

Effect on life span

The effect of ricin on the lifespan of *C. elegans* was tested by incubating early J4 with ricin for 6 h at 21 or 25°C and subsequently transferring five pre-adult juveniles (J4) animals to fresh agar plates seeded with *E. coli*. Animals were transferred to fresh agar plates every 2 days when producing progeny and about once weekly thereafter. Animals were considered dead when they no longer reacted to prodding. Control animals were incubated in PBS only and were processed in parallel with ricin incubated specimens.

Effect on progeny

To determine the effect of ricin on progeny, early J4 stages were incubated with ricin for 6 h at 21 or 25°C. The J4 were then transferred to separate plates and transferred to fresh seeded plates on successive days. Progeny were counted at the adult stage. Unhatched eggs were also counted but have not been included in the totals and accounted for no more than 2% of all progeny.

Micro-injection of ricin A-chain

Micro-injection was performed as described in detail by Mello *et al.* (1991). Ricin A-chain was injected into the distal, syncitial part of the gonad arm. Either both arms or only one arm was injected. Several injection series were carried out. Either the ricin A-chain (Sigma) was injected after dialysis against PBS or it was injected in the buffer in which it was supplied (40% glycerol, 10 mM phosphate (pH 6.0), 0.15 M NaCl, 10 mM galactose, 0.5 mM dithioerythritol). The latter was done because prolonged storage in PBS after dialysation causes precipitation and inactivity of the A-chain (Sigma). Control worms were injected with the corresponding buffer without ricin A-chain. Considering the syncitial organisation of the distal gonad arm, if the ricin was effective against the ribosomes, degeneration and sterility would be the expected result.

Transmission electron microscopy (TEM)

Embedding in Spurr resin was according to Van De Velde and Coomans (1989). LR White fixation was performed according to a protocol provided by N. Kershaw (MRC-LMB, Cambridge, 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 and 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 were 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 polymerisation at 50°C for 24 h.

80 nm thick sections were made using a Reichert OMU-2 ultramicrotome. For specimens embedded in Spurr, formvar coated single slot copper grids were used. For LR White embedded specimens, formvar coated single slot nickel grids were used. Sections were poststained with an LKB ultrostainer for 30 min in uranyl acetate at 40°C and 5 min in lead stain at 20°C. The electron microscope was a Siemens Elmiskop 1A (Siemens, Bad Neuheim, Germany), operating at 80 kV. Nematodes incubated with ricin for 1, 6 and 12 h were used for TEM.

Immunohistochemistry

Whole mount staining

Nematodes that had been incubated for 1, 6 or 12 h in ricin were used. The protocol used was kindly communicated to us by B. Podbilewicz (MRC-LMB). Freeze-cracking was carried out as described above. The slide was dipped in pre-cooled methanol (−20°C) for 5 min and then in acetone (−20°C) for 5 min. The slides were rinsed in PBS for 10 min, followed by PBS containing 0.5% Tween 20 (PBS-Tween). Fixed worms on slides were incubated in PBS-Tween 20 supplemented with 2% dried milk for 15 min to reduce background. All the antibodies were diluted in PBS-Tween-milk. Nematodes on slides

Table 1. *Effect of ricin (50 μg/100 μl) on life span and number of progeny of* Caenorhabditis elegans *at 21 and 25°C (means ± standard deviation, n = 25). Tests with ricin and controls were carried out in parallel.*

	Lifespan (days)		Progeny	
	21°C	25°C	21°C	25°C
Control	19 ± 1	15 ± 1	302 ± 23	150 ± 10
Ricin	19 ± 2	14 ± 2	297 ± 22	156 ± 13

were incubated with a polyclonal rabbit anti-ricin antibody (Sigma) (diluted 1 : 30) overnight at 4°C. The slides were rinsed three times for 5 min in PBS-Tween. The worms were next incubated in FITC-labelled goat anti-rabbit IgG (Sigma) at a 1 : 500 dilution for 45 min at room temperature. Nematodes on slides were then rinsed three times for 5 min each in PBS-Tween and once in PBS, for 5 min, briefly immersed in distilled water, and mounted with a coverslip as described above.

TEM sections

LR white sections were incubated three times for 5 min in PBS, once for 15 min in PBS, 0.2% Tween 20 and subsequently for 30 min in PBS-Tween-milk. The same primary polyclonal anti-ricin antibody and dilution above was used and incubated overnight at 4°C. Sections were washed three times for 15 min in PBS-Tween-milk and incubated in 10 nm gold labelled goat anti-rabbit IgG (Sigma) at a 1 : 50 dilution overnight at 4°C. Sections were washed three times for 15 min in PBS-Tween and three times 15 min in PBS. A final thorough washing was done in PBS and three times in distilled water to prevent salt crystallisation upon drying. Post-staining was carried as described above. Control for specificity of binding was carried out by omitting the primary antibody.

Acid phosphatase histochemistry

Acid phosphatase (AP) staining for TEM was done according to Zhang *et al.* (1991). Nematodes were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) with 8% sucrose at 0°C for 1 h and washed in the same buffer overnight. The nematodes were subsequently incubated in buffer containing acid phosphatase substrate (0.1 M acetate (pH 5.0), 1 mM B-glycerophosphate (Sigma), 2 mM $CeCl_3$, 5 mM sucrose) for 1 h. This buffer was prepared just before use and filtered through a Millipore filter with a 0.2 μm pore size. Nematodes were rinsed three times for 15 min (fresh buffer every 5 min) in incubation buffer without substrate. After rinsing, nematodes were postfixed in OsO_4 and embedded in Spurr as described. Control consisted of incubation in buffer without the substrate B-glycerophosphate.

Acridine orange staining nematodes

Because the acid phosphatase reaction results in a massive, dark precipitation in lysosomes, it proved impossible to unambiguously distinguish the gold label. Clokey and Jacobson (1986) had determined that several dyes (such as acridine orange) are internalised and sequestered in lysosomes in *C. elegans*. Furthermore, Robbins *et al.* (1964) had studied the ultrastructural appearance of cells loaded with AO. They determined that loading with acridine orange led to dark precipitation and appearance of 'myelin membranes' in lysosomes upon counterstaining, making detection of gold label easier. Nematodes were washed off agar plates and rinsed three times in PBS pH 7.2. Acridine orange (AO) was dissolved in PBS and sonicated extensively to break up small amounts of aggregates that did not solubilise. Insoluble material was removed by centrifugation at 10 000 rpm for 1 min in a microfuge. The final dye concentrations was 0.001% for AO (concentration is approximate since a small amount was lost during centrifugation). Incubation was done at 22°C and after 24 h the nematodes were removed and washed five times in PBS.

Results

Exposure of *C. elegans* J4 to 500 μg/ml ricin for 6 h at 21 or 25°C had no detectable effect on lifespan or progeny production (Table 1).

To directly assay the cellular toxicity of the ricin A chain in *C. elegans*, this protein was microinjected into the cytoplasm of syncitial distal gonad. The results of the different injections are summarised in Table 2. In those animals injected with the ricin A-chain the nematode developed a 'hollow-gonad' phenotype (Fig. 1B) with complete degeneration of the gonad. In these injected gonads, no cellular structure could be observed, and no progeny were produced. When one gonad was injected with ricin A-chain and the other with only buffer, the A-chain injected arm degenerated, while the other arm remained healthy and produced progeny (Fig. 1A).

Initial attempts to determine the pathway of gold labelled and unlabelled ricin by tracing ricin uptake using polyclonal anti-ricin antibody and epifluoresence microscopy produced only very weak staining of the head and buccal cavity, too weak to photograph properly (data not shown). Ricin uptake was therefore followed using

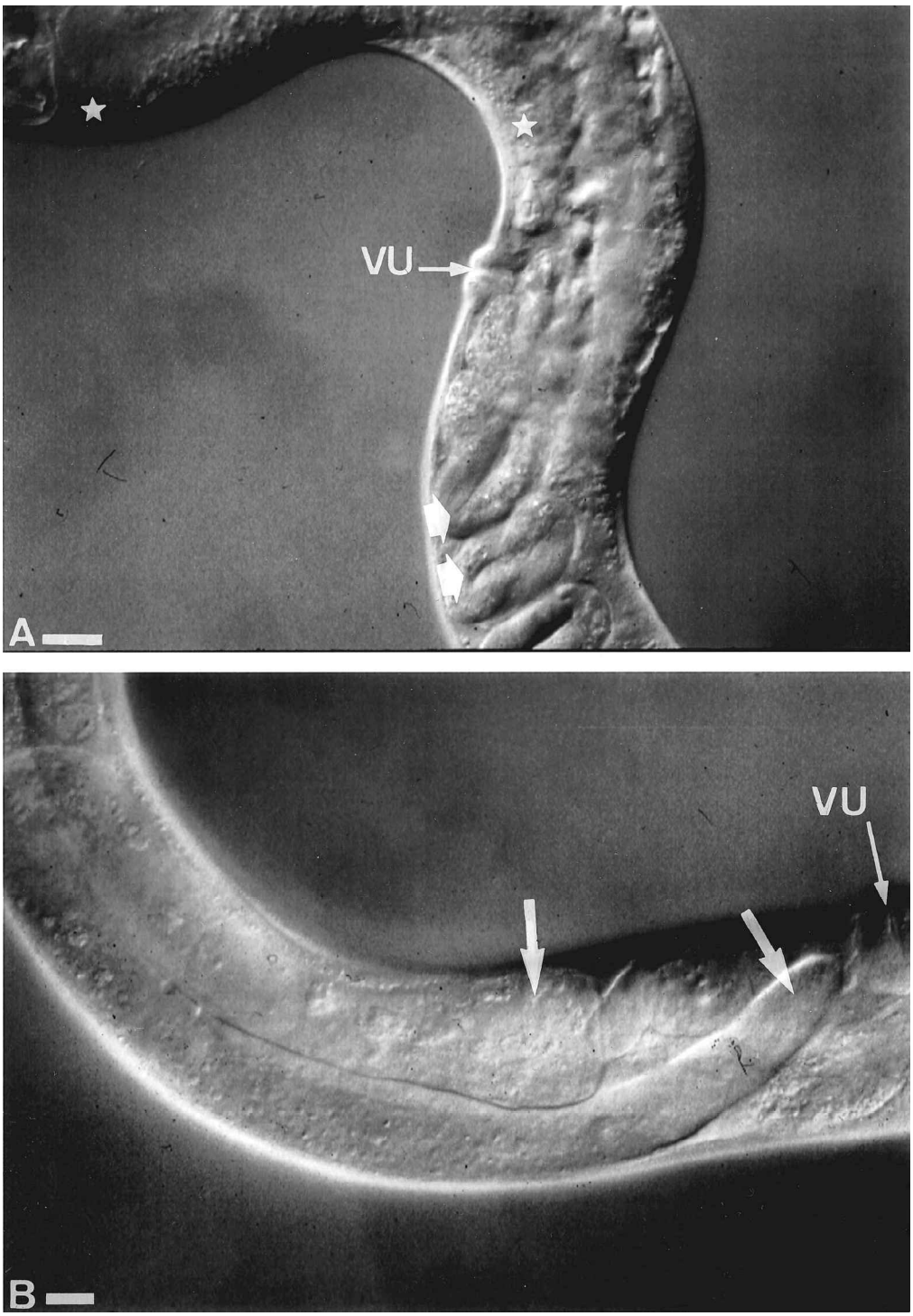

Fig. 1. *Micro-injection of ricin into the gonad of* Caenorhabditis elegans*. A: Hermaphrodite injected in anterior gonad only with ricin A-chain in Sigma buffer; injection resulted in a deformed anterior gonad with no embryos (star) and a normal posterior gonad containing pretzel stage embryos (arrows), anterior left; B: Specimen injected with ricin A-chain in PBS buffer; injection resulted in a hollow gonad phenotype (arrows), only anterior gonad shown; anterior right (VU, vulva; bar = 20 μm).*

TEM, predominantly on sections of animals after 12 h of ricin exposure.

When *C. elegans* was incubated with 5 nm gold labelled ricin, large amounts of label were localised in the intestinal lumen (Fig. 2A, B). However, little label was localised in the intestinal cells. Furthermore, the label appeared in large clumps in the lumen of only few, large multi-vesicular bodies and the individual gold particles are difficult to distinguish (Fig. 2C, D). No label could be identified in any other tissue present (gonad, epidermis, muscles).

When the nematodes were incubated with the native toxin and subsequently traced with a polyclonal anti-ricin antibody, much more label was present in the intestinal lumen (Fig. 3A). Ricin was traced in electron-lucent vesicles and appears in two different states. In some of these vesicles the label was confined to the cell membrane (Fig. 3B, C). In a minority of the vesicles the label was localised in the lumen and on the cell membrane (Fig. 4A, B). With the native toxin, label was also found in huge quantities in the pseudocoelom and the reproductive system (Fig. 4B).

In both the prelabelled and unlabelled incubations, no label was found either in or around the Golgi apparatus, endoplasmic reticulum or in the cell cytoplasm. The ultrastructure of the cells did not reveal any damage or altered structure of the cell organelles in comparison with untreated animals. In contrast to the whole mount labelling, internalisation of the ricin (pre- and unlabelled) was observed in all the intestinal cells studied, including the most anterior ring of four intestinal cells.

To determine the specificity of the internalisation, inhibition of uptake was attempted by preincubating the ricin with 0.1 M lactose. In a control, the gold labelled ricin was mixed with a fivefold concentration of native ricin. In both experiments we were able to identify gold label easily in the intestinal lumen, but not in the intestinal cells.

In order to identify the intestinal vesicles containing the gold label, nematodes were stained for acid phosphatase (Fig. 4C-E). Of the several electron-lucent vesicles present in the intestinal cytoplasm, several contained the typical dark precipitation (Fig. 4D). This was not observed in the control (Fig. 4C). This dark precipitation prevented identification of the gold label. Using acridine orange as a lysosomal marker allowed simultaneous identification of gold label and lysosomes (Fig 4E). Staining with acid phosphatase and acridine orange revealed that in both cases electron-lucent vesicles contained gold label.

TEM revealed the presence of gold label in developing embryos (data not shown). Since the label was predominantly present in embryos already encased in an eggshell preventing fixative from entering the embryo, tissue quality was poor. Therefore, the possibility of the presence of gold label being the result of smear during cutting of the tissue was possible.

Discussion

Microinjection into the gonad resulted in degeneration in those arms where the A-chain was injected resulting in sterility. This raises the possibility that *C. elegans* ribosomes are also sensitive to depurination. Furthermore, sequence data of *C. elegans* (Wilson *et al.*, 1994) shows the target of ricin to be present in *C. elegans* rRNA.

The strong and specific *in vitro* binding of ricin to the intestinal brush border indicates the presence of binding sites. However, this does not lead to toxicity when *C. elegans* is incubated in ricin concentrations of up to 500 μg/ml, as no cellular damage or decrease in life span or progeny was evident at this high level of exposure.

Although the nematodes processed for TEM were incubated in large amounts of ricin and the intestinal lumen contained considerable amounts of label, the intestinal cells contained relatively little label. This might be explained by paucity of binding sites, but is more likely reflective of uptake processes.

The different appearance of the labelled (groups) and unlabelled (at random) toxin in the intestinal lumen cannot be readily explained but is most likely due to the physical constraints imposed by the 5 nm gold label. Data to support this interpretation is the observation by Van De Velde (1984) that BSA labelled 20 nm gold does not enter the glycocalyx between the microvilli in *C. elegans* while BSA-FITC enters the cytoplasma readily (Clokey & Jacobson, 1986) in the same species. This would explain the relatively predominant appearance of the prelabelled toxin at the tips of the microvilli and the rather sparse amount between the villi, in comparison with the native toxin.

We have been unable to localise the effective endocytosis. The slow internalisation of the ricin resulted in only a few small vesicles observed near the cell membrane containing gold label. Furthermore, the presence of the well developed and dark terminal web, immediately underlying the cell membrane, made identification of the endocytosis more difficult. The use of a whole animal makes any evaluation of retro-endocytosis (the process of trans-

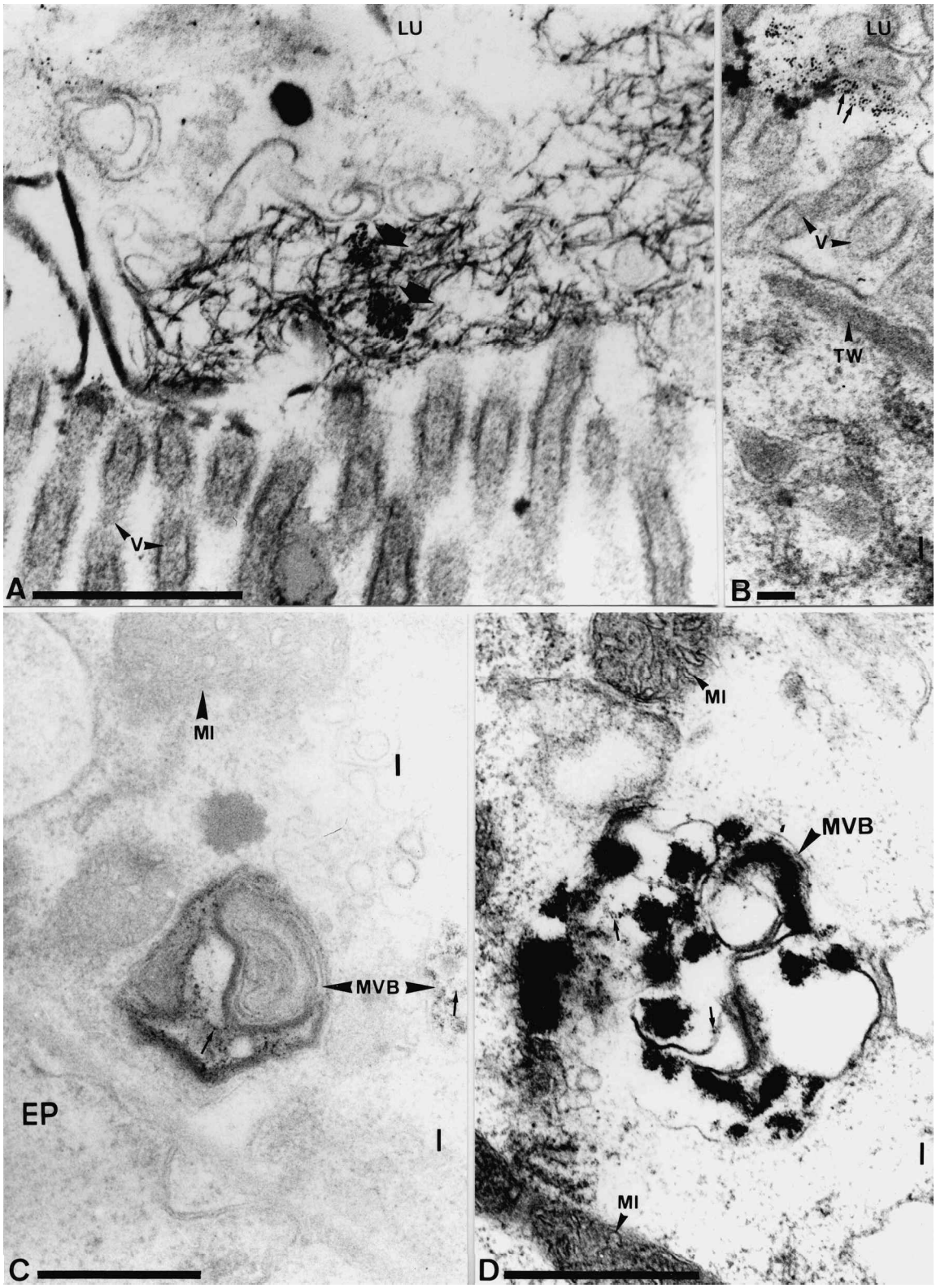
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Table 2. *Micro-injection of ricin A-chain into the distal part of the gonad of* Caenorhabditis elegans *(Sigma: buffer in which the ricin A-chain was delivered). Stock concentrations: test 2, 100 μg/ml; tests 4, 5, 400 μg/ml.*

Test	Injection buffer	Number injected	Gonads injected	Number surviving	Gonad structure	Progeny
1	PBS	5	2	4	normal	normal
2	PBS+ricin	10	2	3	degenerated	none
3	Sigma	10	2	6	normal	normal
4	Sigma+ricin	8	2	3	degenerated	none
5	Sigma+ricin	2	1 (anterior)	2	injected gonad degenerated, other normal	none normal

port back out of the cell) unreliable. The time needed to thoroughly rinse the intestinal lumen free of ricin would have depleted the amount being retro-endocytosed.

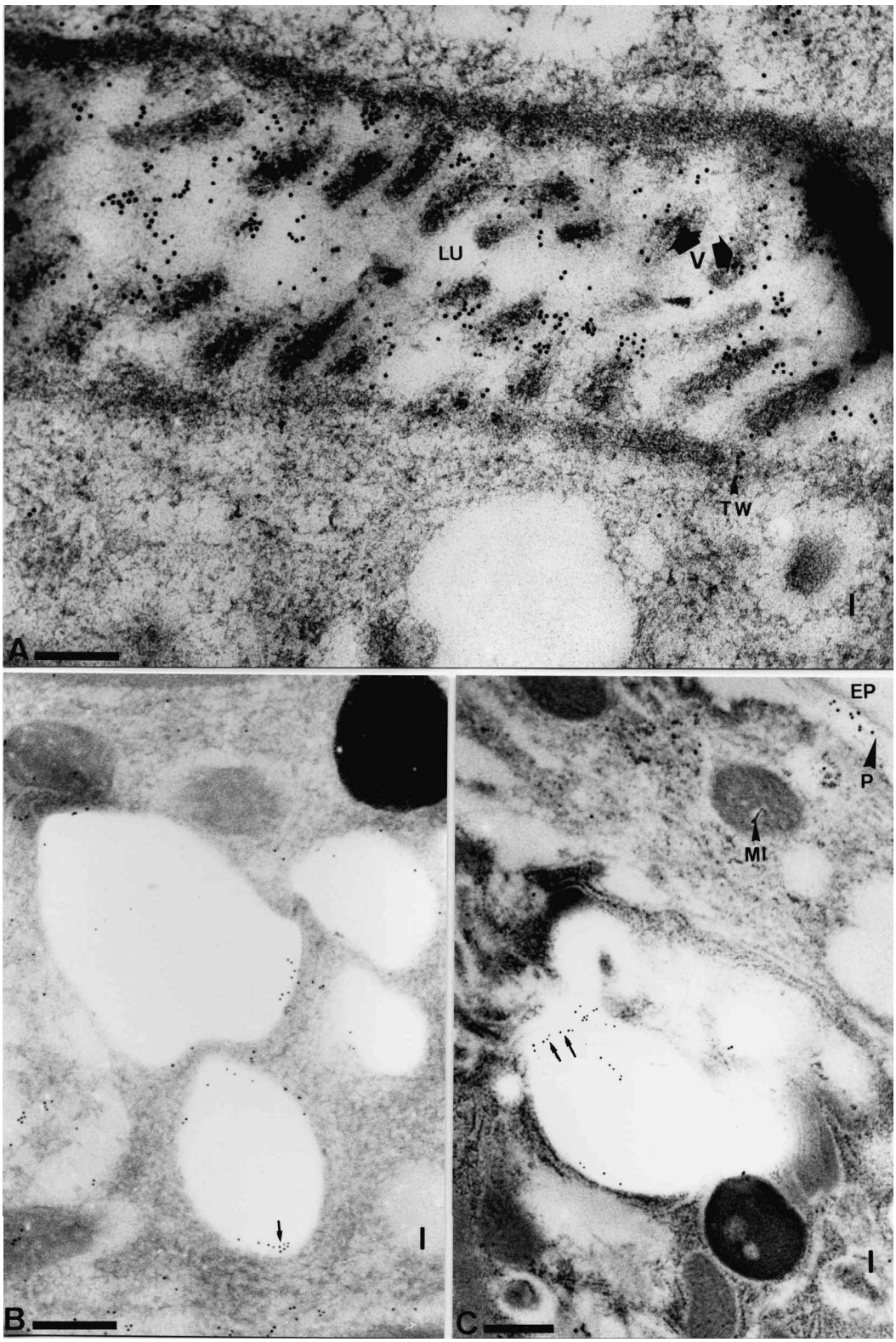
The difference in intracellular sorting between prelabelled and unlabelled toxin has been reported (Van Deurs *et al.*, 1986). The valency of the label (influencing binding properties) would be the determining factor causing different routing. The possibility that the 5 nm gold became detached from the toxin and followed a different pathway is unlikely since specimens fed on prelabelled toxin and subsequently analysed with a polyclonal anti-ricin antibody confirmed the multivesicular body localisation.

The pathway of the native toxin, which is the most authentic (not influenced by attached label), reveals considerable differences. At least two different types of intestinal vesicles harbour ricin. One of these is a lysosome, evident by the positive acridine orange staining. Furthermore, only in those vesicles is the majority of the gold label found in the vesicle lumen, the most likely place for digestion to take place in a lysosome. In the non acridine orange staining vesicles the gold label is predominantly found very close to the inner side of the vesicle membrane.

Although no data is available concerning the kind of receptors responsible for internalisation, these non acridine orange staining vesicles, containing membrane-bound label most likely point towards galactosyl specific endocytosis (Magnusson *et al.*, 1991, 1993). The affinity of the ricin B-chain to galactosyl residues is not markedly affected by the low pH in endosomes. Van Deurs *et al.* (1990) showed that even at pH 5.0 there is little dissociation of bound ricin to galactosides, in contrast to binding of ricin to mannose receptors (Brech *et al.*, 1993). Supporting this supposition is the observation that lactose proved to be an effective inhibitor of endocytosis in this study; this would not be expected in the case of mannose mediated endocytosis.

The vesicles, containing the toxin still bound to the membrane, are likely to be involved in the transcytosis observed towards the pseudocoelom. We have been unable to unambiguously determine the exact place of toxin entry into the reproductive system. Considerable amounts of label were observed in embryos already encased in an eggshell. Far less label was observed in oocytes or embryos in early stages of development, but because of the damage to the gonadal tissue due to the fixation problems with eggs already encased in an eggshell, the observed label in oocytes and embryos early in development could be an artefact, *e.g.*, the result of a smear effect. This could indicate that the toxin would be internalised late in development. Alternatively, the label could be incorporated continuously, but at a low rate. Analysis of the sections from specimens that were incubated for 12 h reveals that very little label is present in the lysosomes in any given

Fig. 2. *Intestinal lumen of* Caenorhabditis elegans *incubated for 12 h with 5-nm gold prelabeled ricin holotoxin. A: Aggregates of gold label (arrowheads); no staining is readily visible between the intestinal microvilli; B: More dispersed staining in the intestinal lumen; C, D: Typical appearance of multi-vesicular body exhibiting large amounts of internalised label; individual gold label (small arrows) is not as distinct as in the intestinal lumen; in any given intestinal cell only one or two such multi-vesicular bodies were observed; the multi-vesicular bodies and their content were not observed in control incubations; picture in C was deliberately underexposed to improve resolution of label (I, intestine; LU, intestinal lumen; MI, mitochondrion; MVB, multi-vesicular body; TW, terminal web; V, intestinal microvilli; scale bars A, C, D = 0.5 μm, B = 0.1 μm).*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cell when unlabelled toxin is used. When incubated with unlabelled toxin, more toxin seems to be transcytosed than transported to the lysosomes. This might explain the absence of a positive result when using the PAb for the whole mount staining of nematodes incubated with ricin. The small amount present at any time most likely was beyond the resolution of that technique.

The observation of free ricin in the pseudocoelom and its subsequent incorporation in embryos without causing any toxic effect is surprising. One possible explanation would be to assume that the subunits become dissociated during the intracellular routing, thereby considerably reducing the possibility of toxic action. Because the polyclonal antibody used recognises not only the holotoxin but both separate subunits as well, it is impossible to determine whether such a process does occur and the secreted ricin is holotoxin or not. The only cases where identification can be done is in Figs 3C, D and 4A where the appearance of peripheral gold label is indicative of receptor binding to the surface of the vesicles. Since the B-chain is responsible for binding, this observed labelling pattern could represent either the B-chain only or the holotoxin, but is most unlikely to represent the A-chain.

In contrast to the whole mount staining with the lectin, indicating no binding sites on the anterior ring of four cells, TEM shows that the ricin is also internalised in these cells. It is possible that binding sites are indeed present but that the difference of fixation employed in both staining techniques has an effect on these binding sites. The use of acetone-only fixation in whole mounts would have a significant impact if the binding sites consist of glycolipids. However, no clear explanation is currently available.

The toxicity of ricin towards *C. elegans* cells indicates that ricin would be a suitable candidate for use in cell ablation. Constructing transgenic strains containing the ricin A-chain under control of an inducible promoter, would allow precise destruction of cells or tissues at certain points during development. Such an approach using ricin A-chain has already successfully been used in transgenic mice (Breitman *et al.*, 1987; Landel *et al.*, 1988), *Drosophila* (Moffat *et al.*, 1992) and *Dictystelium* (Shaulsky & Loomis, 1993).

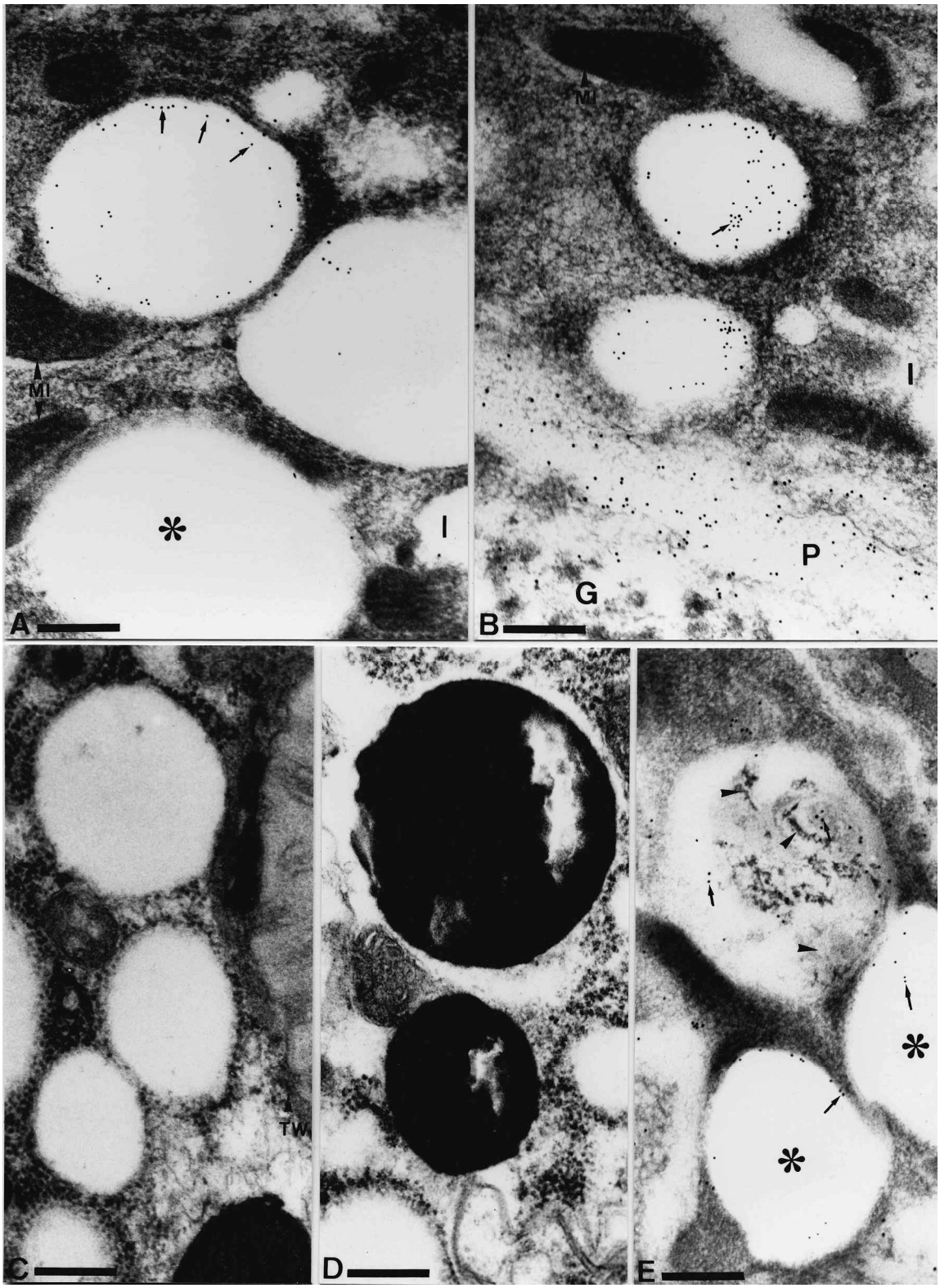
Resistance of intact cells to ricin, despite sensitivity of their ribosomes in a cell-free system, has been reported before. Maruniak *et al.* (1990) reported that cultured cells from the insects *Trichoplusia ni* and *Spodoptera frugiperda* are resistant to the holotoxin, although their ribosomes are sensitive to depurination by ricin. However, the study contained no data about the mechanism. The internalisation of a toxin without eliciting toxicity has been observed before with Shiga toxin in A431 cells (Sandvig *et al.*, 1992). In the present study, TEM strongly suggests that the non-toxicity is due to altered sorting of the ricin, such that the toxin does not reach the Golgi apparatus considered essential for the translocation of the A-chain into the cytoplasm (Lord *et al.*, 1994; Sandvig & Van Deurs, 1994). Although the TEM technique cannot prove the complete absence of ricin in the trans Golgi network, these observations contrast dramatically with those of comparable cell culture studies, where ricin is readily detected in the trans Golgi network.

As observed for work done on cell lines (Sandvig & Van Deurs, 1994) it is evident from the TEM observations that ricin binds opportunistically to many receptors on the cell surface. However, the results presented here clearly show that internalisation of ricin-bound receptors does not necessarily lead to toxicity.

It has been reported that the delivery of ricin to the TGN is a temperature sensitive process, inhibited at temperatures of 18-20°C (Van Deurs *et al.*, 1987). The possibility that temperature could be a contributing factor in the resistance is excluded. The reported temperature induced inhibition operates at 18-20°C. None of the experiments was carried out below 21°C. Furthermore, label was consistently observed in lysosomes, which would not be likely in the case of a temperature induced transport inhibition.

This observed non-toxicity is surprising. Considering the catalytic mode of action of the A-chain and its well documented efficiency in killing cells, it requires that the pathway followed must be extremely leak-proof, the slightest error resulting in cell death. It is recognised that such errors might be present but are difficult to detect in this experimental system since the loss of even a few cells does not necessarily result in a dead nematode

Fig. 3. *Intestinal lumen of* Caenorhabditis elegans *incubated for 12 h with native ricin holotoxin and visualised using immunohistochemistry. A: At random appearance of gold label when native ricin is used and visualised using 10-nm gold; B, C: Several intestinal vesicles of which only the electron-lucent contain membrane bound label (small arrowheads); C: Vesicle containing membrane bound label and indication of gold label present in the pseudocoelomatic cavity (I, intestine; LU, intestinal lumen; MI, mitochondrion; TW, terminal web; V, intestinal microvilli; scale bar = 0.25 μm).*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as evidenced by the widespread use of cell destruction using laser ablation in *C. elegans* research (Wood, 1988). Nevertheless, we did not observe signs of cellular decay or gold label present at the Golgi or the RER in any of the sections studied, indicating correct sorting of the toxin. It is currently unclear in what structure the toxin or separate subunits are internalised and stored in the embryo so as to avoid toxicity and why the epidermis does not contain any indication of incorporation.

Of two other nematodes tested but not included in the present study, one (*Panagrolaimus superbus*) exhibited strong *in situ* staining along the entire intestinal tract, but was not sensitive to ricin. A second species (*Acrobeloides maximus*) did not exhibit either *in situ* staining nor any sensitivity to ricin (Borgonie *et al.*, 1994). All three nematode species mentioned feed on bacteria. Furthermore, the nematode phylum is one of the oldest invertebrate phyla known (Vanfleteren *et al.*, 1990). It is also known that several bacteria produce toxins, and at least Shiga toxin has an identical mode of action to ricin. It is conceivable that more ricin or Shiga-like toxins are produced by bacteria and that consequently throughout evolution a constant selection pressure has been present on bacteriophagous nematodes selecting for resistance.

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Fig. 4. *Intestinal cells of* Caenorhabditis elegans *incubated for 12 h with native ricin holotoxin and visualised using immunohistochemistry. A: Vesicles containing membrane bound label and one vesicle (asterisk) devoid of label; B: Vesicles containing membrane bound ricin (small arrowheads) and ricin predominantly present in the vesicle lumen (small arrows); also evident is considerable label present in the pseudocoelom; it is doubtful if the random label present in the gonad is authentic; C-E: Lysosome identification using acid phosphatase and acridine orange; C: Electron-lucent vacuoles in* C. elegans *intestinal cells, section from control specimen incubated in acid phosphatase buffer minus the substrate – glycerophosphate, no precipitation evident; D: Section of specimen stained for acid phosphatase, asterisks indicate vesicles staining positive for AP; this precipitation made identification of gold label impossible; note that not all vesicles exhibit precipitation; E: Section of specimen incubated with acridine orange and subsequently with native ricin for 12 h; two types of vesicles are evident; the acridine orange induced dark deposits in lysosomes (arrowheads) together with label predominantly present in the vesicle lumen (small arrows); second type of vesicles is electron-lucent (large arrows) and the label is bound to the cell membrane (small arrows) (I, intestine; TW, terminal web; V, intestinal microvilli; P, pseudocoelom; G, gonad; scale bars = 0.25 μm).*

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