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Effect of nematicidal *Bacillus thuringiensis* strains on free-living nematodes. 2. Ultrastructural analysis of the intoxication process in *Caenorhabditis elegans*

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Summary – Transmission electron microscopy is used to describe the intoxication in *Caenorhabditis elegans*, feeding on toxic spore/crystals of *Bacillus thuringiensis*. The toxin acts directly against the intestine, first by affecting the anteriormost ring of four intestinal cells. Over a period of 12 hours, these cells lose much of their volume, the microvilli regress slowly, several cell organelles undergo dramatic change and are ultimately destroyed. No rupture of the apical intestinal cell membrane is observed. Non-intestinal tissues seem unaffected. This study indicates considerable ultrastructural differences in the mode of action between the nematicidal toxin and the insecticidal crystal toxins from *B. thuringiensis*.

Résumé – ***Effets de souches nématicides de* Bacillus thuringiensis *sur les nématodes libres. 2. Analyse ultrastructurale du processus d'intoxication chez* Caenorhabditis elegans** - La microscopie électronique par transmission a été utilisée pour décrire l'intoxication de *Caenorhabditis elegans* se nourrissant sur des spores/cristaux de *Bacillus thuringiensis*. La toxine agit directement sur l'intestin où elle affecte initialement l'anneau de quatre cellules le plus antérieur. En 12 heures, le volume de ces cellules diminue considérablement, les microvillosités régressent lentement, de nombreux organites cellulaires subissent des changements spectaculaires pour être finalement détruits. Il n'a pas été observé de rupture de la membrane cellulaire apicale. Les tissus autres qu'intestinaux n'apparaissent pas affectés. Cette étude révèle des différences ultrastructurales considérables entre le mode d'action des toxines nématicides et celui des cristaux insecticides émanant les uns et les autres de *Bacillus thuringiensis*.

Key-words : *Bacillus thuringiensis, Caenorhabditis elegans,* pathology, TEM.

A preliminary characterization of nematicidal *Bacillus thuringiensis* (Bt) strains was reported (Borgonie *et al.*, 1996). These preliminary results indicated that some fundamental differences were evident between the nematicidal and the better characterized, insecticidal Bt strains. To detail the extent of differences and gain more insight into the nematicidal mode of action, the current study reports on the ultrastructural effects of the intoxication process in the nematode *Caenorhabditis elegans* during the first 12 h of intoxication at the level of the anteriormost ring of four intestinal cells.

Materials and methods

PREPARATION OF SPORE-CRYSTAL MIXTURES OF *B. THURINGIENSIS*

One non-nematicidal (BTS0302AE) *B. thuringiensis (Bt)* strain was obtained from the collection of Plant Genetic Systems NV (stored in 25 % glycerol at – 70 °C), and one nematicidal (Edwards *et al.*, 1989) *B.t.* strain (NRRL repository No. B-18247) from Northern Research Laboratory, U.S. D.A., Peoria, Illinois, U.S.A. Both strains were grown on 400 ml CBI (Culturing Bacillus Isolates) medium in 2-1 Erlenmeyer flasks on a rotary shaker (100 rpm) at 28 °C for 5 to 7 days. Composition of the CBI medium : bacto-peptone 7.5 g; glucose 1 g; K_2HPO_4 4.35 g; distilled water to 1 l. After adjustment to pH 7.2 and sterilisation at 120 °C for 20 min, two filter-sterilized salt solutions were added : $MgSO_4 \cdot 7H_2O$ 2.46 g; $MnSO_4 \cdot H_2O$ 0.04 g; $ZnSO_4 \cdot 7H_2O$ 0.28 g; $FeSO_4 \cdot 7H_2O$ 0.4 g; distilled water to 100 ml and $CaCl_2 \cdot 2H_2O$ 3.66 g; distilled water to 100 ml. Upon lysis, vegetative cells, spores, and crystals were harvested by centrifugation (3000 rpm) for 15 min. The pelleted particles, consisting mostly of spores and crystals were resuspended in PBS (phosphate buffered saline : NaCl 8 g; KCl 0.2 g; Na_2HPO_4 1.15 g; KH_2HPO_4 0.2 g; distilled water to 1 l; pH 7-8) and stored at a concentration of 2.10^9 particles/ml at – 20 °C until use.

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Nematodes

Two free-living nematode species were used which belong to two families within the Rhabditida : *Caenorhabditis elegans* var. Bristol (Rhabditidae) and *Acrobeloides maximus* (Cephalobidae).

Monoxenic and axenic culture

All cultures were sterilized 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 of *C. elegans* were kept at 20 °C and cultured axenically according to Vanfleteren *et al.* (1990). Briefly, a mixture of 3 % (w/v) soy peptone and 3 % (w/v) yeast extract were autoclaved under standard conditions. A 0.1 % hemoglobin solution was aseptically added. The hemoglobin solution was made by dissolving 5 g hemoglobin (SERVA) in 100 ml of 0.1 M KOH and autoclaved for no longer than 10 min. Culture in an axenic medium was preferred in this study of the intoxication process by Bt to avoid contamination from *E. coli*.

Bacillus thuringiensis incubation

Incubation of nematodes in spore-crystal mixtures was done by adding 50 µl aliquots of spore-crystal mixtures to wells of a 96 well microtiter plate (Falcon) that contained 50 µl suspensions of 200 nematodes in PBS. Control wells consisted of 50 µl nematode suspensions in PBS supplemented with 50 µl PBS. The non-toxic *B.t.* strain 302AE was used as control. Nematode suspensions contained tetracyclin (30 µg/ml) and chloramphenicol (30 µg/ml) to prevent spore germination and subsequent bacterial growth. Microtiter plates were incubated for 1, 6 and 12 h at 25 °C after which young, still living females were selected for TEM processing.

Transmission electron microscopy

Young females were collected in distilled water and fixed at room temperature in Karnovsky (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 fixative minus acrolein, and nematodes were cut in half. They were 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 h in 2 % OsO_4 in 0.2 M Na-cacodylate buffer pH 7.2 at 4 °C. Nematodes were transferred to 50 % alcohol and rinsed several times until the black colour disappeared and subsequently transferred to 2 % uranyl acetate in 50 % alcohol and stained for 1 h. Dehydration was done using 70, 90 and 100 % alcohol (three times 20 min each) at room temperature. Nematodes were infiltrated with resin using 100 % alcohol/Spurr (1:1) overnight and then transferred to absolute alcohol/Spurr (1:2) for 8 h. In the final transfer to 100 % Spurr the samples were left overnight at 4 °C. Polymerization occurred at 70 °C for 12 h.

Sections of 80 nm thickness were cut using a Reichert OMU-2 Ultramicrotome. Formvar coated single slot copper grids were used. Nematodes were sectioned at three sites along the intestinal tract : immediately posterior to the pharynx, at midbody, and anterior to the anus. Sections were poststained with an LKB ultrastainer for 30 min in uranylacetate at 40 °C and 5 min in lead stain at 20 °C. Electron micrographs were made using a Siemens Elmiskop 1A, operating at 80 kV. The following nematodes were sectioned : *C. elegans* N2 that fed on *B. thuringiensis* NRRL-18247 and on *B. thuringiensis* 302AE, and *A. maximus* that fed on *B. thuringiensis* NRRL-18247. All incubations and sectionings were repeated twice to confirm observations. Only young adult hermaphrodites were used in the present study.

Results

With T.E.M., no ultrastructural changes were observed in the anteriormost ring of four intestinal cells of nematodes that fed for one hour on the toxic spore-crystal mixture derived from the nematicidal strain NRRL-18247 (Figs 1B; 2A, D, G) when compared with nematodes incubated only with PBS (Figs 1A, B; 2A, D, G).

Changes were more obvious after 6 h feeding on the toxic *B. thuringiensis* strain (Figs 1C; 2B, E, H). The most prominent changes were the extensive widening of the intestinal lumen, considerable loss of intestinal cell volume, and the regression of microvilli (Fig. 2). The glycocalyx between the microvilli remained clearly visible and still formed a barrier; no spores or crystals were observed between microvilli (e.g. Fig. 2H). Mitochondria stained darker than those in controls and almost without exception were rounded, and contained cristae with considerably widened lumens (Fig. 2E). Although the nucleus remained intact and bounded by a double nuclear membrane which is clearly visible and seemingly unaffected (Fig. 2H), the nucleolus at times contains a few spherical vacuole-type structures. The terminal web (TW), rough endoplasmic reticulum (RER), and Golgi apparatus appeared visibly intact and unaltered. The intestinal cells contained numerous lipid, yolk and electron-lucent vacuoles. The cytoplasm acquired a patchy appearance and sometimes contained a few crystal-like inclusions, that differed from the control or the nematodes fed with the non-toxic strain of *Bt*. The intestinal lumen was filled with spores and crystals. Crystals of various shapes and sizes were observed *in vitro* but only pieces of crystal were observed in the lumen (Figs 2B; 3C).

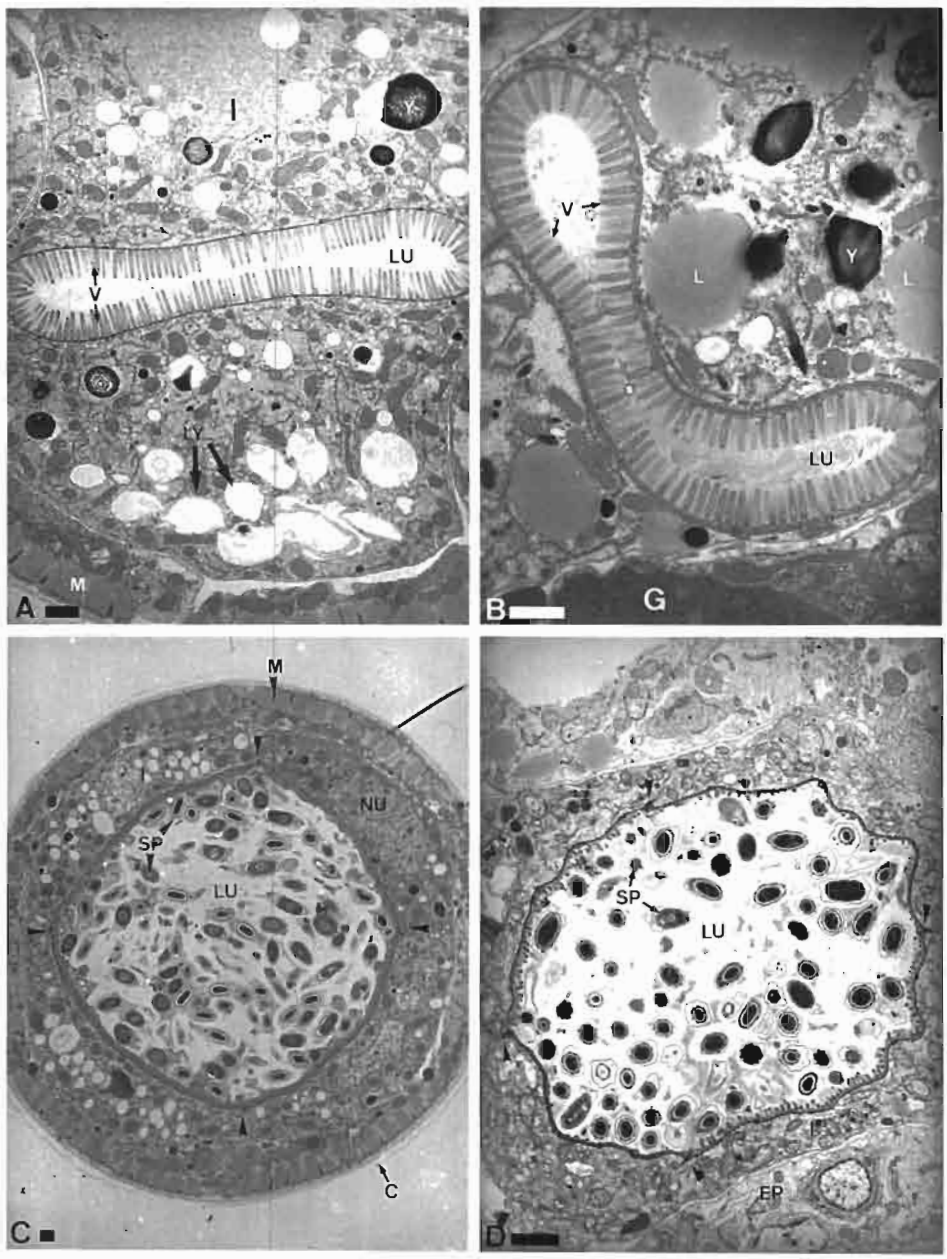

Fig. 1. *T.E.M. of anterior intestine showing increasing damage as intoxication proceeds.* Caenorhabditis elegans *fed on nematicidal* Bacillus thuringiensis *strain NRRL-18247 at 25 °C. A : Control, no spores and crystals added; B : Anterior intestine, 1 h after intoxication; C : Anterior intestine, 6 h after intoxication, dilatated lumen filled with spores; D : Anterior intestine, 12 h after intoxication* (Arrowheads indicate the position of the four belt desmosomes. Bar = 1 μm).
Abbreviations. *LY : lysosomes; LU : intestinal lumen; L : Lipid vacuoles; Y : yolk vacuole; I : intestinal cell; V : microvilli; M : body muscle; G : gonad; C : cuticle; NU : nucleus; SP : spores; EP : epidermal cell; I : intestinal cell; CR : crystal; NO : nucleolus; TW : terminal web.*

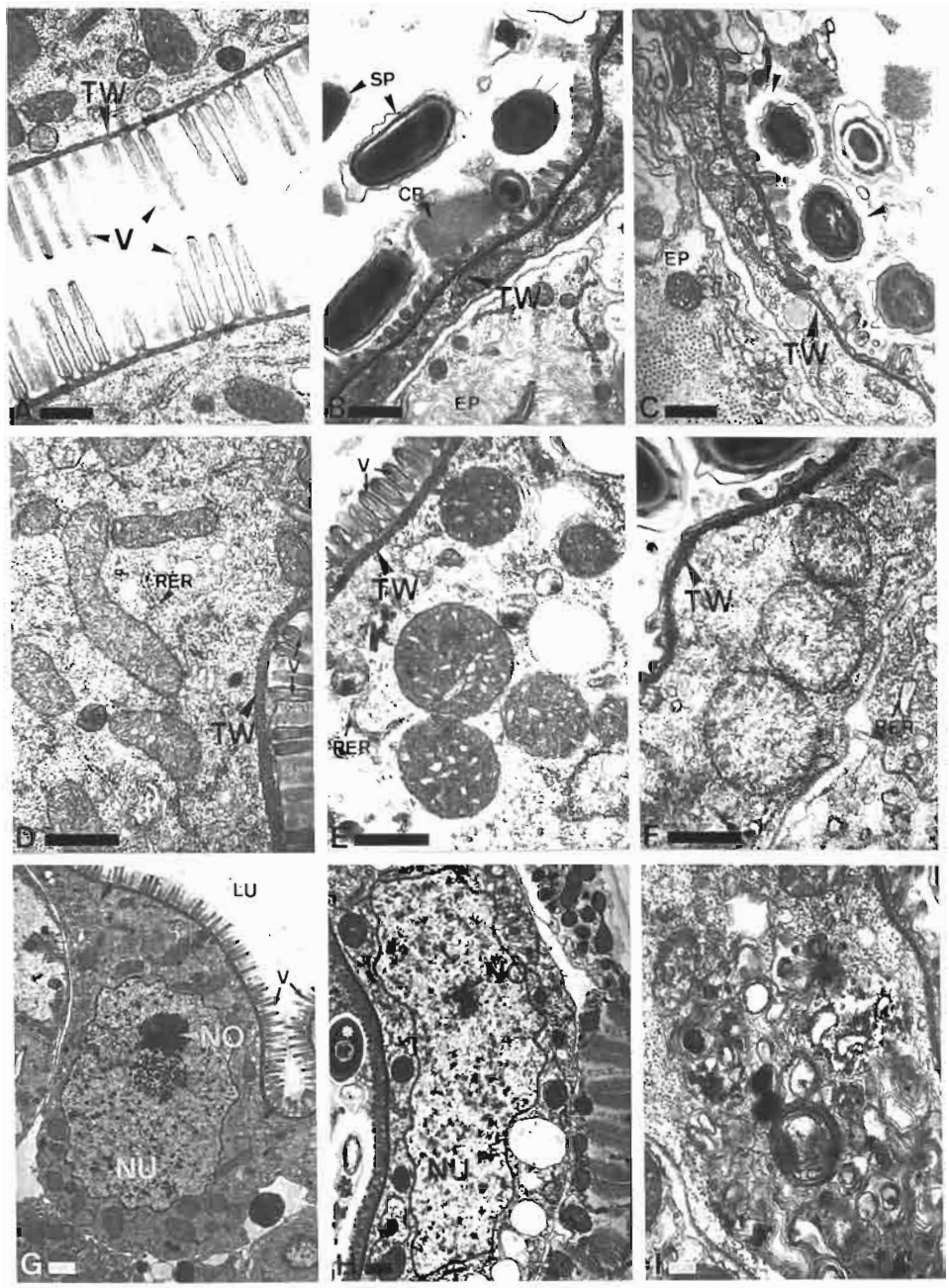

Fig. 2. *Progressive destruction of cell organelles in anterior ring of four cells in* Caenorhabditis elegans *fed on* Bacillus thuringiensis *NRRL-18247. (A, D, G : after 1 h intoxication; B, E, H : after 6 h intoxication; C, F, I : after 12 h intoxication). A-C : Progressive recession of microvilli (arrowheads point to often observed "halo" around spores); D-F : Progressive rounding of mitochondria, becoming increasingly electron-lucent (note the unraveling and thinning of the terminal web as the intoxication proceeds); G-I : Nuclear structure unaltered at 1 and 6 h, at 12 h no nucleus could be identified with certainty, structure visualised is most probably remnant of one of the nuclei. White asterisk (2H) shows spore firmly on top of the microvilli. Spores were never observed to penetrate between the microvilli* (Bar = 0.5 μm).
Abbreviations : *see Fig. 1.*

Twelve hours after introduction of the toxic spore-crystal mixture, the previously described phenomena became more clearly pronounced and spread to other, previously unaffected organelles (Fig. 1D). As the lumen widened, intestinal cells decreased in volume, leaving in some places only residual cytoplasm, barely surrounding the mitochondria. The apical intestinal cell surface became irregular. The microvilli regressed to the extent that they were no longer visible (Fig. 2C, F) in some regions of the intestinal lumen. The TW became thinner subjacent to these regions. The mitochondria lumen was electron-lucent (Fig. 2F) and in some cases showed lysis of the mitochondrial cell membrane. The most striking feature of intestinal cell damage was the loss of nuclei. A systematic search of some sections revealed no intact nuclei, although one structure was identified which may have been a remnant of one nucleus (Fig. 2I). Also prominent was the large reduction of vacuolar compartments that are usually present in large numbers in control cells. TW and Golgi apparatus seemed unaffected as were yolk and lipid vacuoles (data not shown). Although the RER seemed intact, the lumen was sometimes considerably wider than in animals that fed on the non-toxic spore-crystal mixture (Fig. 2D-F). However, the degree of lumen dilation varied considerably. No lysis of apical intestinal cell membrane was observed and the lateral intestinal membranes were intact as well.

The remainder of the intestine (midlevel and posterior) and tissues other than the intestinal cells were unaffected up to 12 h after ingestion of the toxic strain. The only visible alterations after twelve hours of feeding were the increased transparancy and rounding of mitochondria. The cuticle remained intact and seemed at all times unaffected, which was also the case for body wall muscles and epidermal cells.

The accumulation of spores and crystals in the anterior intestine was not observed in *C. elegans* fed on the non-toxic strain 302AE of Bt (Fig. 3A) or in a non-sensitive nematode *(A. maximus)* fed on *B. thuringiensis* strain NRRL-18247 (Fig. 3B).

In the intestine, *B. thuringiensis* spores and crystals were sometimes surrounded by a distinct "halo" (Fig. 2C). An amorphous mass that occurred between the spores and crystals closely resembled the glycocalyx in healthy animals. Although crystals are formed during sporulation in *B. thuringiensis*, only a very small number (pieces) of crystals were identified (Fig. 3C). The several layers of the spore coat were readily discernable and seemed unaffected. In a few instances spores and crystals could be seen in close proximity with, or in actual contact to the cell membrane of intestinal cells.

No attempts were made to make sections of intestinal tissue after more than 12 h of intoxication. After 12 h, damage was so extensive and it was not clear whether observations showed parts of a dead cell rather than pathological effects of the toxin.

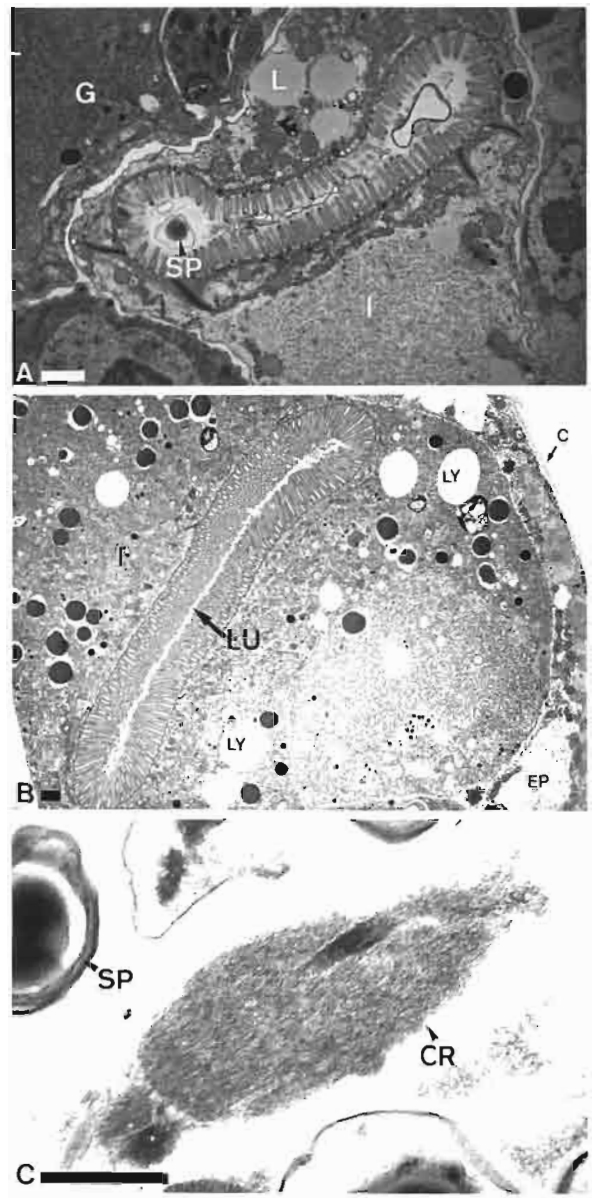

Fig 3. *A, B : Control animals, anterior intestine. A :* Caenorhabditis elegans *fed for 12 h on the non-nematicidal strain* Bacillus thuringiensis *302AE. Only very few spores could be identified; B :* Acrobeloides maximus *fed for 12 h in the presence of* B. thuringiensis *strain NRRL-18247. No widening of the lumen or regression of villi was observed; C : Remnant of a partially digested crystal of strain NRRL-18247 in the lumen of* C. elegans *12 h after intoxication.* (Bar equivalent : A, B = 1 µm; C = 0.25 µm).

Abbreviations : *see Fig. 1.*

Discussion

Although the effect of nematicidal *B. thuringiensis* on animal-parasitic nematodes has been reported (Bone *et al.*, 1985; Bottjer & Bone, 1987), to our knowledge, no detailed ultrastructural description of this effect has yet been reported.

In this study, ultrastructural observations show that the first phase of toxicity from *Bt* spores/crystals is characterized by the gradual destruction of the anterior intestinal ring of four cells immediately posterior to the pharynx. Damage to the remainder of the intestine or to non-intestinal tissues, if present, is not visible, except for rounding of mitochondria and widening of RER cisternae.

When the intoxication process in *C. elegans* is compared to what has been reported in insects, where a huge body of data is available (Gill *et al.*, 1992; Knowles & Dow, 1993) some analogy is observed, but considerable differences are evident.

An important difference with insecticidal endotoxins is that the intoxication process in nematodes takes hours to exhibit the drastic changes observed, much longer than when *B. thuringiensis* is used against insects (Knowles & Dow, 1993). However, a considerably slower mode of action has been reported for *B. thuringiensis* var. San Diego on the cottonwood leaf beetle (Bauer & Pankratz, 1992), where the first effects are visible after 2 h.

In insects, intoxication of midgut cells leads in minutes to : loss of the basal involutions; swelling of the apical microvilli; vesiculation of the RER, loss of ribosomes; and swelling of mitochondria, cell and nucleus with subsequent rupture of the nuclear, organelle and plasma membranes. This ultimately leads to release of cell contents into the intestinal lumen (Gill *et al.*, 1992; Knowles & Dow, 1993). Toxic *Bacillus sphaericus* extracts or purified toxin proteins rapidly cause almost identical intoxication phenomena, although there are indications that the *B. sphaericus* toxin is internalized into the intestinal cells (Davidson & Titus, 1987). In *C. elegans*, the intoxication process is different in both toxins. Intestinal cells lose much of their volume. Microvilli in *C. elegans* also disappear but, whereas this loss in insects is the result of cell swelling, in *C. elegans*, microvilli slowly regress more or less uniformly. The surprising feature is the regression of the central axis of the microvilli. Concurrently, the width of the strongly developed TW is reduced by approximately one half. Although the constituents of the central axis of the microvillus and of the TW are not identical, they share several proteins in common (Weiser *et al.*, 1988). This difference in susceptibility to disruption might become of importance when studying the ultimate target of the nematicidal factor of *B. thuringiensis*.

Since the actin binding protein villin is known to modulate formation and regression of microvilli (Bretscher & Weber, 1979; Matsudeira & Burgess, 1979; Robine *et al.*, 1985; Friederich *et al.*, 1989, 1992), attempts were made to identify changes in the distribution of villin during intoxication using an anti-human villin monoclonal antibody (kindly provided by Dr. E. Friederich, Pasteur Institute, France). This failed because no binding of the antibody was observed in nematode tissues (unpubl. observ.).

In lepidopteran insects, *B. thuringiensis* toxins act at the level of the apical microvilli of columnar cells. Nevertheless, the adjacent goblet cells also undergo dramatic changes because of the existence of gap junctions between both types of cells (Knowles & Dow, 1993). Since no gap junctions have been observed between nematode intestinal cells and there is a highly restricted site of cell destruction, lateral diffusion of the toxin seems highly unlikely. In view of this restriction, the toxin is probably not transcytosed to the pseudocoelomatic cavity where it could affect other tissues.

There is no evidence of cell membrane lysis. Therefore, the massive cellular damage observed is probably not due to the rupture of lysosomal membranes. Indeed, a massive release of hydrolytic enzymes would undoubtedly damage all organelles equally in the immediate vicinity. This was not observed : some organelles were severely damaged (nucleus, mitochondria) while others remained largely unaffected (Golgi, RER).

Peritrophic membrane-like structures (PM) have recently been shown to be present in several bacteriophagous nematodes, including *C. elegans* (Borgonie *et al.*, 1995). While some examples showed that nematodes feeding on poisons led to a massive secretion of PM as a defense reaction (Weiser *et al.*, 1988; Peters, 1992) we did not observe any indication of such a reaction to the nematicidal *B. thuringiensis* strain. It was observed that neither spores nor crystals penetrated the glycocalyx. Instead, they were found on top of the microvilli, indicating that the barrier on top of the microvilli/glycocalyx complex remained intact up to 12 h after intoxication, although intestinal cells were severely damaged.

Despite the fact that during sporulation each vegetative cell forms a crystal besides a spore, very few crystals were ever observed in the lumen of the intestine. The observation of only pieces of crystals after 12 h indicates that the crystals are being dissolved rather slowly in the nematode intestine. However, in the absence of purified toxin, it remains to be determined whether the nematicidal factor is incorporated within the crystal and whether the dissolution of the crystal liberates the toxin. Observations using T.E.M. did not reveal any indication of spore germination in the intestinal lumen prior to death of the intestinal cells, either in the toxic or in the non-toxic *B. thuringiensis* strain, thereby eliminating spore germination, multiplication, and subsequent blocking of the intestine as the cause of death.

TEM indicates that the first four anterior cells are seriously affected 12 h after intoxication, and that these

are the only cells where damage can be observed. In the absence of any indication of damage to any other cells at 12 h after intoxication, it is intriguing to know what ultimately causes death of the nematode within 24 h. There are indications, although without evidence, that massive damage to intestinal cells in *C. elegans* does not lead to death of the nematode in the first few hours (Junkersdorf & Schierenberg, 1992). In two separate experiments, using a laser, they ablated either the progenitor of the anterior intestine (Ea) or the progenitor of the posterior intestine (Ep) in embryos. Ablation of Ea led, in nine out of sixteen cases, to a viable hatching juvenile with only half of the intestine. In the Ep ablation, thirteen out of seventeen cases also hatched with only half of the intestine. The authors reported that in observing further development of thirteen animals in which one of the progenitors was ablated, the animals died in a few days as young larvae (Junkersdorf & Schierenberg, 1992). Although these data show that J1 can survive with half an intestine for several days, one needs to be cautious in extrapolating these results observed in J1 to adults as studied here. Additional effects were reported for *B. thuringiensis* var. *israelensis* insecticidal toxins, with disruption of function of insect muscular and nervous system (Chilcott *et al.*, 1984; Singh & Gill, 1985; Singh *et al.*, 1986). There is evidence for ultrastructural and biochemical differences between the anterior ring of four intestinal cells and the rest of the intestine in *C. elegans* (Beh *et al.*, 1991; Borgonie *et al.*, 1995). If no additional toxin or target for the nematicidal *B. thuringiensis* is present in non-intestinal tissues, one could assume that the anterior ring of four cells occupies a vital function in *C. elegans* and that destruction of these anterior cells might have wider detrimental effects. This assumption has no further supporting evidence to date.

Based on the available data, it is impossible to determine the ultimate target of the nematicidal factor. The observed rounding, swelling and transparency in mitochondria, for example, are often interpreted as signs of stress (Smith & Ord, 1983). However, this effect could be the result of many different intoxication phenomena, and not necessarily an indication that mitochondria are the prime target of the toxin.

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