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Effects of *Bacillus thuringiensis* δ-Endotoxin on Insect Fat Body Structure

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Sequential observations of binding patterns and structural effects of *Bacillus thuringiensis* var. *kurstaki* were made on fat body tissue of the fall webworm, *Hyphantria cunea* Drury. Fat body was cultured *in vitro* in the presence of purified 62 kDa endotoxin and then examined for protein synthesis and the localization of membrane-bound toxin detected by an antibody against the 62 kDa endotoxin. Protein synthesis was mostly inhibited at concentrations of 15 μg/ml and higher. Immunocytochemical observations suggest that the toxin binds to all exposed basal lamina surrounding the fat body without apparent specificity. The cytopathic effect detectable by scanning electron microscope is disintegration rather than cell swelling. The basal lamina bound toxin was eventually detached from the fat body and followed by an extrusion of cell contents like lipid granules.

Bacillus thuringiensis is a gram-positive insect pathogenic bacterium which produces a crystalline protein body in the cell during spore formation. The protein body called δ-endotoxin is a strong toxin to lepidopteran or dipteran larvae. When these protein bodies are ingested by larvae, they are dissolved into active toxin by digestive fluids in the larval midgut (Lecadet, 1970).

The activated toxin exerts a cytolytic effect in midgut and other epithelial cells, leading to cell swelling and epithelial disruption (Endo and Nishiitsutsuji-Uwo, 1980; Lüthy and Ebersold, 1981; Percy and Fast, 1983). The toxin is thought to bind to plasma membrane-associated receptors forming lytic pores (Knowles and Ellar, 1987), although the precise binding mechanism and cytological distribution of binding sites remain obscure.

Histopathological studies, utilizing light and electron microscopic techniques, in various insects reveal the effects of the toxin in midgut cells. *Bacillus thuringiensis* toxin appears to cause a loss of basal involutions, swelling of the apical microvilli, vesiculation of the endoplasmic reticulum with concomitant loss of ribosomes, and the deformation of mitochondria (Angus, 1970; Galani, 1973; Ebersold et al., 1977; Kissinger and McGaughey, 1979; Lacey and Federici, 1979; Endo and Nishiitsutsuji- Uwo, 1980; Griego et al., 1980; Lüthy, 1980; Nishiitsutsuji-Uwo and Endo, 1981).

Binding of activated toxin to membranes of organs other than midgut in insects can be studied by immunocytochemical analysis. Immunocytochemical approach has been used previously in the study of the lepidopteran *Heliothis virescens* (Ryerse et al., 1990). The authors reported nonspecific binding of the *B. thuringiensis* var. *kurstaki* 63 kDa δ-endotoxin to all exposed plasma membranes without apparent specificity for particular membrane domains in *Heliothis virescens*.

In this study, we tested the effects of *B. thuringiensis* var. *kurstaki* toxin on protein synthesis and structure of *Hyphantria cunea* fat body. The results presented here describe the binding of *B. thuringiensis* var. *kurstaki* δ-endotoxin to the membrane of fat body tissue. The activated toxin disrupts cell structure and causes extensive histolysis of fat body cells as it does in the midgut of insects.

Materials and Methods

Experimental animals

Hyphantria cunea (fall webworm) was obtained from a colony maintained in the Insects Resource Laboratory of the Institute of Biotechnology, KIST. Larvae were reared on artificial diet at 26℃ and 75% relative humidity with a photoperiod of 16 h light and 8 h dark.

Bacteria and toxin preparation

Bacillus thuringiensis var. *kurstaki* HD-1 was obtained from Dr. R. Faust (USDA, ARS, Beltsville, Maryland). Growth and sporulation conditions were maintained as described by Kim et al. (1993). The method used for crystal δ-endotoxin purification was described by Thomas and Ellar (1983). Crystalline δ-endotoxin was purified by ultracentrifugation on discontinuous sucrose gradients. Crystals were incubated for 60 min at 37℃ in 50 mM-Na_2CO_3/HCl (pH 9.5) and 10 mM-dithiothreitol (DTT). Insoluble material was removed by centrifugation at

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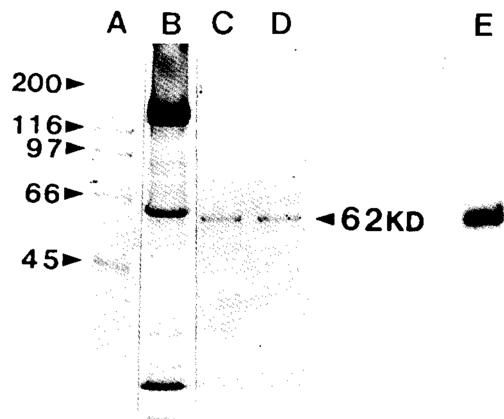

Fig. 1. SDS-PAGE (A-D) and immunoblots (E) of crystal proteins of *Bacillus thuringiensis* var. *kurstaki* HD-1. Lane A, molecular weight standards: myosin 200 kDa, β-galactosidase 116 kDa, phosphorylase B 97 kDa, bovine serum albumin 66 kDa, ovalbumin, 45 kDa. Lane B, 30 μg of the undigested crystal protein. Lanes C and D, 40 μg solubilized crystal protein treated with trypsin for 1 d (C) and 2 d (D) as described in the Materials and Methods. Lane E, 20 μg of purified 62 kDa endotoxin separated by SDS-PAGE, transferred to nitrocellulose paper, and incubated with the antibody as described in the Materials and Methods.

10,000 g for 5 min and the supernatant was dialized against the 10 mM Tris-glycine buffer (pH 8.3).

The solubilized crystal protein in 10 mM Tris-glycine buffer (pH 8.3) was proteolytically activated *in vitro* using trypsin (TPCK-treated, Sigma) as described by Nagamatsu et al. (1984). Tryptic digestion produced 62 kDa endotoxin with one or two minor peptides (Fig. 1). For further purification of 62 kDa endotoxin, large slab gels (170 mm × 180 mm × 3 mm) were used. Electrophoresis was conducted using Tris-glycine buffer without SDS or 2-mercaptoethanol. After electrophoresis, the toxin band was excised, eluted, and dialized against Ringer's solution (125 mM NaCl, 1.8 mM $CaCl_2$, 1.3 mM KCl, pH 7.3). Protein concentrations were determined using Bradford (1976) procedure with bovine serum albumin (BSA) as the protein standard. Toxin preparations were routinely checked for protein homogeneity by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) using a 12.5% separating slab gel. Representative preparations are shown in Fig. 1.

In vitro toxin exposure and tissue processing

Fat bodies used in the *in vitro* culture were dissected from 5-day-old 7th instar larvae. The tissues were exposed to 62 kDa endotoxin in Grace's medium at 0.5, 1.0, 1.5, 2.0 and 2.5 μg/100 μl for 1 h with agitation at room temperature. The tissues were then labeled in the same medium containing [^{35}S]-methionine at 250 μCi/ml for an additional 1 h for protein synthesis. Proteins were prepared from washed fat bodies by sonication in fat body homogenization buffer (50 mM Tris-HCl, pH 8.0, containing 1 mM PMSF, 1 mM dithiothreitol, 1 mM EDTA and 0.01% [w/v] 1-phenyl-2-thiourea) and saved as a post-12,000 g supernatant of tissue sonicates.

For microscopic studies, dissected fat bodies were exposed to 62 kDa endotoxin in Grace's medium at 2.0 μg/100 μl for 30, 60, and 120 min with agitation at room temperature. The tissues were then washed 3 × 10 min in PBS, and further fixed in proper fixative for microscopic study.

Immunofluorescence

The fat bodies were fixed in 4% paraformaldehyde in PBS for 2 h at 4℃. The fixed tissues were rinsed overnight in PBS containing 20% sucrose and frozen-sectioned with 10 μm thickness using a cryostat (American Optics) at -20℃. Each section was then mounted on gelatin coated slides and stored at -80℃ until use.

Immunolabeling was performed as described by Cowley et al. (1994). Tissue sections on slides were air-dried, washed twice in PBS for 5 min, and permeabilized in 0.2% Triton X-100 in PBS for 15 min. The specimens were then quenched in 100 mM glycine in PBS for 10 min, followed by 3% BSA in PBS for 30 min. Primary antibody incubations were performed at a dilution of 1 : 200 in PBS containing 0.1% Tween-20 for 1 h at room temperature. After a 3 × 15 min wash in PBS, FITC-conjugated goat anti-rabbit IgG (Sigma) was used at a dilution of 1 : 150 in PBS containing 0.1% Tween-20 for 1 h at room temperature. After washing in PBS, sections were mounted in PBS containing 90% glycerol and 1 mg/ml ρ-phenylenediamine (Sigma). In one set of controls, immunofluorescence was carried out in the preimmune serum in the presence of secondary antibody. In another, immunofluorescence was performed in the presence of primary antibody but in the absence of the secondary antibody. In all cases, the observed immunofluorescence signals were dependent on the presence of specific primary and secondary antibodies.

Immunocytochemistry

Immunocytochemistry was performed as previously described (Miller et al., 1990). Fat bodies were fixed for 3 h in a mixture of 4% formaldehyde and 1% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.5) containing 0.15 mM $CaCl_2$ and 0.45 M sucrose (FM). Fixation was continued overnight in pH 10.4 FM lacking glutaraldehyde. Rinsed tissues were dehydrated in a graded ethanol series (up to 95%) and embedded in lowicryl K4M (Polysciences). Ultrathin sections mounted on formvar-coated gold grids were treated for 10 min with Tris-buffered saline (TBS; 0.02 M Tris-HCl, pH 7.5, containing 0.5 M NaCl, pH 7.5) followed by 3% BSA for 30 min in TBS, and then for 2 h in TBS containing antiserum against 62 kDa endotoxin at a 1 : 200 dilution. Following a wash in TBS containing 0.3% BSA, the sections were incubated in a 1 : 5 dilution of

gold-conjugated goat anti-rabbit IgG (20 nm: Zymed) for 60 min. The grids were finally washed with TBS and distilled water, and examined using a Hitachi H-600 transmission electron microscope.

Gold particles observed in controls were typically few in number and unlocalized. Controls included (1) substitution of preimmune serum for primary antiserum, (2) use of secondary antibody in the absence of treatment with primary antiserum, and (3) treatment of thin sections with colloidal gold alone.

Gel electrophoresis and autoradiography

SDS-PAGE was performed according to the method of Laemmli (1970). Homogenate protein was determined by the method of Bradford (1976) with bovine serum albumin as the standard, and an equal amount of protein was applied to 12.5% slab gels. Samples were dissolved in SDS-PAGE sample buffer (0.65 M Tris-HCl, pH 6.8, containing 2% SDS, 5% mercaptoethanol, and 0.01% bromophenol blue) and boiled for 3 min.

Following electrophoresis, the gel was stained in 0.25% Coomassie brilliant blue in 40% methanol and 10% acetic acid for 3 h, and destained in 30% methanol and 5% acetic acid. Destained gels were vacuum-dried for autoradiography. Autoradiograms were prepared by exposing dried gels on X-ray film for one week.

Western blot

Following SDS-PAGE, proteins in the gel were electrotransferred to a sheet of nitrocellulose (0.2 µm, Bio-Rad). The blots were blocked in TBS containing 5% nonfat dry milk and then incubated with antiserum against 62 kDa endotoxin at 1 : 1000 dilution in TBS. After washing, the blots were incubated with peroxidase-conjugated anti-rabbit IgG antibody (Bio-Rad) at 1 : 3000 dilution in TBS for 1 h. Immunoreactivity was determined using the ECL chemiluminescence reaction (Amersham).

Preparation of antiserum

Preparation of antiserum was performed as described by Kim et al. (1989). Purified 62 kDa endotoxin (500 µg/ml) was mixed with an equal volume of Freund's complete adjuvant (0.5 ml) and was injected subcutaneously into rabbits three times every other day with a fourth injection given 1 wk later. Booster injection (0.5 ml protein and 0.5 ml Freund's incomplete adjuvant) was given 2 wk after the fourth injection. Blood was collected 1 wk after the fifth injection, allowed to coagulate at 40 ℃ overnight, and centrifuged at 10,000 g for 10 min. For the purification of IgG, the supernatant was applied to protein-A-sepharose column (ImmunoPure IgG purification Kit, Pierce) as recommended by supplier.

Scanning electron microscopy

Fat bodies were fixed with 2.5% buffered glutaraldehyde for 2 h, using 0.1 M phosphate buffer pH 7.5. The tissues were then washed several times with the same buffer. Postfixation was done with 1% buffered OsO_4 for 1 h at 4℃. Thereafter, the samples were dehydrated in the ethanol series, treated with propylene oxide, critical point dried in CO_2, mounted, and sputter gold coated (Brandt et al., 1978; Cohen, 1974). Samples were observed using a JSM 6400 operated at 15 KV.

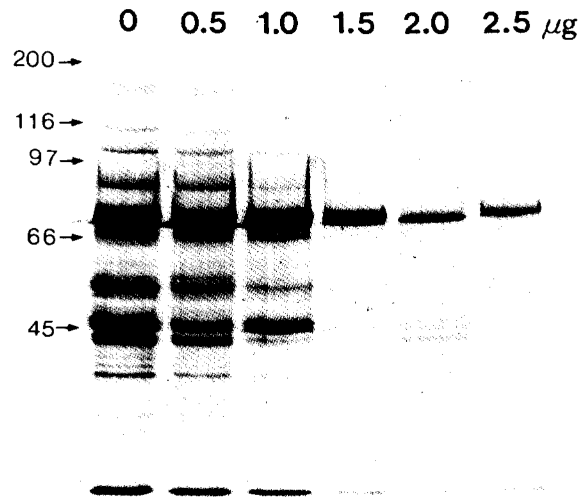

Fig. 2. Protein synthesis of fat body tissue in the presence of 62 kDa endotoxin. Toxin concentrations for *in vitro* culture are indicated in µg/100 µl. The [^{35}S]-methionine-labeled proteins from individual toxin-treated and control fat bodies were separated on SDS-PAGE, and visualized by autoradiography. Approximately 20 µg of protein were applied per well.

Results

Protein synthesis of fat body exposed to 62 kDa endotoxin

Fig. 2 shows the autoradiograph obtained after the extracts of radiolabeled tissues that were run on a 12.5% acrylamide SDS gel. The fat bodies were radiolabeled *in vitro* in the presence of 62 kDa endotoxin. As the toxin concentration increased up to 10 µg/ml, a gradual decrease of protein synthesis was observed. Above 15 µg/ml of endotoxin, protein synthesis was completely inhibited, except for one or two bands (Fig. 2). All subsequent binding experiments with endotoxin were carried out at the concentration of 20 µg/ml.

In vitro binding of 62 kDa endotoxin to fat body tissue

62 kDa endotoxin was applied to fat body to perform binding studies. The bound 62 kDa endotoxin was revealed by immunofluorescence and immunogold labeling using antibody (Figs. 3 and 4). In tissue exposed to endotoxin for 5 min or 15 min, there were very weak binding signals (unpub. data). After 30 min exposure, immunostaining apparently showed a positive reaction along the surface of fat body tissues. By high magnification electron microscopy, it was confirmed that gold

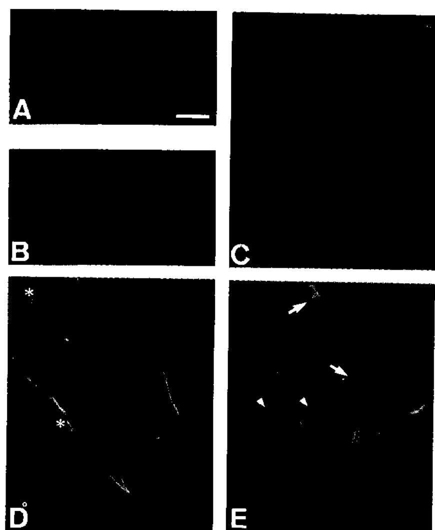

Fig. 3. Immunofluorescent micrographs of fat body tissue incubated with *Bacillus thuringiensis* var. *kurstaki* 62 kDa endotoxin and subsequently with antibody against 62 kDa endotoxin (B-E) or preimmune serum (A), and stained with fluorescein-conjugated secondary antibody. B, control; C-E, 30 min, 60 min, and 120 min after toxin exposure; asterisk, basal lamina beginning to separate from fat body tissue; arrows, tissue debris; arrowheads, basal lamina separated and disrupted. Bar=50 μm.

(Fig. 4C, arrow), while at other locations no gold particles were attached yet (Fig. 4C, arrowhead). At this cellular level, fat body cells were found to be surrounded by basal lamina to which endotoxins bind. The cell organelles, like mitochondria and rER, were almost normal. The binding pattern looked homogeneous by fluorescent microscopy (Fig. 3C) but a little heterogeneous by electron microscopy (Fig. 4C).

After 1 h toxin exposure, a high degree of staining was observed throughout the membrane by immunofluorescence (Fig. 3D). During this exposure time, homogeneous binding pattern was observed, and the basal lamina partially began to separate from the fat body tissue (Fig. 4E). Histolysis was visible, leaving an unclear culture medium. This detached location detectable by electron microscopy seems to be consistent with the thick staining site observed by immunofluorescence (Fig. 3D, asterisk). Generally, the provacuoles (Fig. 4, asterisk), which are an example of a particular kind of plasma membrane recycling (Locke, 1984), seem to disintegrate before disruption of the cell organelles. The environmental changes resulting from contact between toxins and membrane might affect provacuoles in short time.

By 2 h after exposure, membrane structure was extensively destroyed, leaving tissue debris (Figs. 3E, 4G, arrows). During this time, fat bodies were broken down to pieces too fragile to keep as samples, making the culture medium very turbid (unpub. data). The cytopathic change detectable with electron microscopy is an abnormal structure of rER and mitochondria (Fig. 4F arrow, Fig. 4G, arrowheads) as those reported after toxin ingestion or injection (Endo and Nishiitsutsuji-

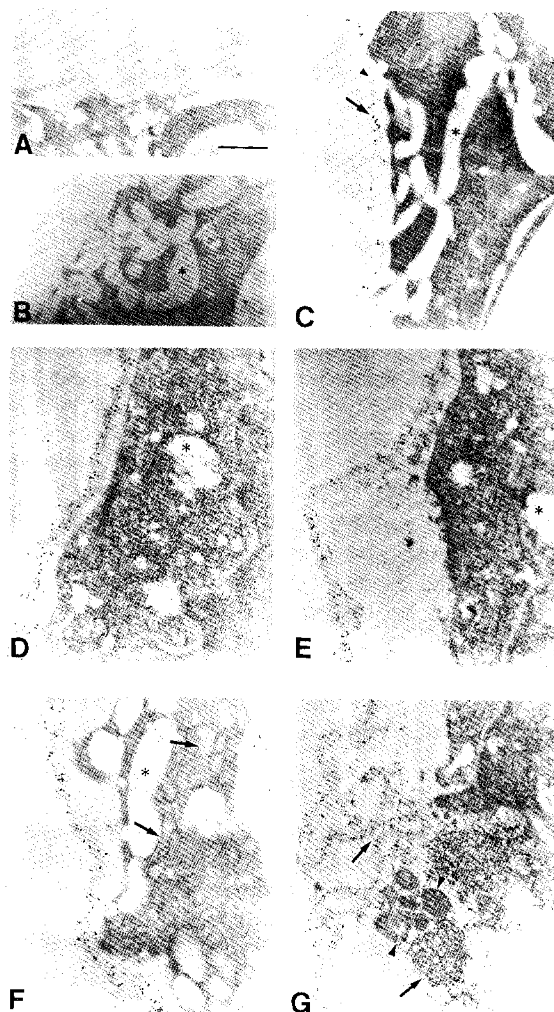

Fig. 4. Immunolocalization of 62 kDa endotoxin in fat body tissue cultured *in vitro* in the presence of 62 kDa endotoxin. Antibody against 62 kDa endotoxin (B-G) or preimmune serum (A) were applied and then labeled with goat-anti rabbit IgG-gold as described in Materials and Methods. B, control; C, 30 min after toxin exposure. At some membrane areas gold particles are concentrated (arrow). D-E, 60 min after toxin exposure. Panel D shows basal lamina labeled by gold particles still surrounding tissue. Panel E shows basal lamina is separated from tissue with the same exposure time as panel D. F-G, 2 h after toxin exposure. The spherical form of rER (panel F, arrow) and abnormal shape of mitochondria (arrowheads) are observed. Extensive decay is accompanied by cell debris (panel G, arrows) around tissue. * ; provacuole. Bar=1 μm.

Uwo, 1981; Percy and Fast, 1983).

In the control fat body, the basal lamina was not distinguishable from the plasma membrane (Fig. 4B). Whereas in toxin-exposed fat body, the basal lamina began to separate and transformed from a very thin layer (Fig. 4C) to a thick swollen layer (Figs. 4D-G). However, it was not obvious whether basal lamina separated together with the plasma membrane from tissue.

Surface change of fat body tissues by 62 kDa endotoxin

To investigate surface changes of fat body, tissues

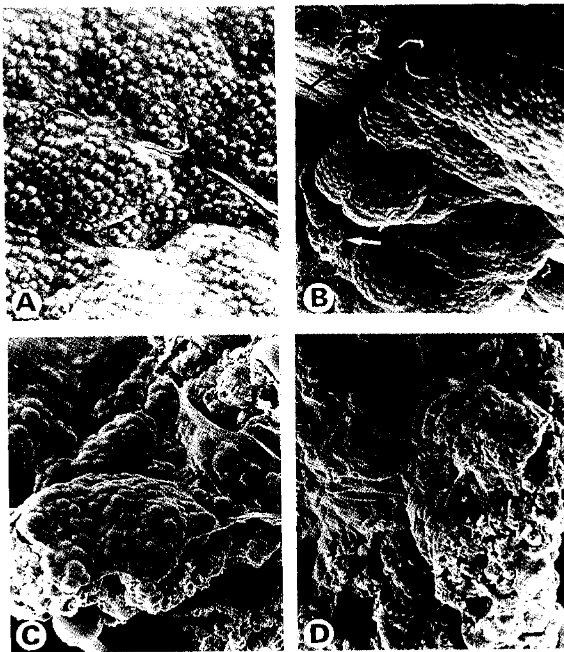

Fig. 5. Scanning electron micrograph showing fat body tissue exposed to 62 kDa endotoxin for 30 min (B), 60 min (C), and 120 min (D). Panel B shows two locations (arrows) beginning to decay. A, control. Bar=10 μm.

were monitored by scanning electron microscope. The results provided an unexpected phenomenon. The control fat body is shown in Fig. 5A. It consists of a sheet of cells full of lipid granules. The 30 min-toxin-exposed fat body appeared nearly normal, however two locations showed minute rupture (Fig. 5B, arrows). By 1 h after exposure, endotoxin had caused extensive damage to the surface of fat body tissues. Some locations still had surrounding basal lamina like normal tissue, while other regions showed destruction, leading to extrusion of cytoplasmic contents containing lipid granules. As exposure time increased up to 2 h, cytopathological alterations became more severe, involving extensive structural disorganization and shredding (Fig. 5D). The toxin-exposed fat body tissues appeared to shred rather than swell.

Discussion

The present study has shown that *B. thuringiensis* var. *kurstaki* HD-1 δ-endotoxin binds to insect fat body, inhibits protein synthesis, and induces tissue decay in an *in vitro* system. The concentration of 62 kDa endotoxin necessary to inhibit *in vitro* protein synthesis was over 15 μg/ml, which is several times greater than those reported from other *in vitro* systems (Nishiitsutsuji-Uwo et al., 1979; Johnson and Davidson, 1984; Himeno et al., 1985; Reisner et al., 1989). One explanation for the requirement of higher toxin concentration is that the basal lamina surrounding the fat body may retard the access of toxin to the plasma membrane. This was confirmed by a report that the presence of basal lamina which surrounds the tubular epithelium might result in a different sensitivity between serosal (basal) and mucosal (luminal) exposure to endotoxin (Oschman and Berridge, 1971; Caulfield and Farquhar, 1974). Other possibilities are the differences in toxin preparation, tissue type, and the susceptibilities of insect species. Purified endotoxin peptide seems to show weak toxicity compared to that of the crude mixture (Bauer and Pankratz, 1992).

The inhibition of protein synthesis by endotoxin might result from the vesiculation of endoplasmic reticulum concomitant with loss of ribosomes which is a common pathological action of *B. thuringiensis* on larvae of lepidopteran insects (Nishiitsutsuji and Endo, 1981; Percy and Fast, 1983; Lane et al., 1989). The binding of *B. thuringiensis kurstaki* endotoxin to fat body tissue has been shown by immunofluorescent staining and immunogold labeling.

By short-term exposure, endotoxin binds discontinuously to the basal lamina of fat body when examined by electron microscope. However, with the passage of time, the entire membrane was saturated with endotoxin along the surface of fat body tissue, suggesting that the components in the membrane that interact with toxin are evenly distributed.

Recently, the site of toxin binding *in vivo* has been demonstrated directly by immunocytochemical analysis of ingested toxins (Bravo et al., 1992). This study reports that CryIIIA bound preferentially to the apical microvilli of the posterior part of the midgut in the coleopteran, *L. decemlineata*. It has also been reported that the toxin from *Bacillus sphaericus* shows higher pattern for the posterior part of the midgut in some dipteran insects (Davidson, 1989).

In contrast, another immunocytochemical approach has been reported in the lepidopteran *Heliothis virescens* (Ryerse et al., 1990). They reported nonspecific binding of the *Bacillus thuringiensis* var. *kurstaki* HD-73 δ-endotoxin to all exposed plasma membrane, and the apparent continuity of toxin binding along the apical microvillar membranes of the midgut and Malpighian tubule cells. These observations both in midgut and in Malpighian tubules are consistent with our observation that toxin binding occurs uniformly along the membrane. We did not find any regional or developmental difference in toxin binding in fat body tissue (unpub. data). Regional differences or different binding patterns (Davidson, 1989; Bravo et al., 1992) might result from variation in host species, tissue type, toxin preparation, and procedure of toxin exposure.

The difference in toxin distribution on membrane after short-term exposure and in cytological alteration observed by scanning electron microscopy could be due to the contact chance between toxin and membrane during *in vitro* culture. The amount of disruption varies, even in adjacent cells, with apparently healthy cells being found in the immediate vicinity of those severely affected (Percy and Fast, 1983). This suggests

that direct contact of the cell with the toxin is probably required and that no lateral intercellular diffusion of cytoplasmic response occurred (Percy and Fast, 1983). The toxin might exert its effect on the closest available membrane regardless of the cellular location of the membrane.

By covalently binding toxin to Sephadex beads which are too large to penetrate a cell, and then bioassaying the bound toxin in tissue culture cells, Fast et al. (1978) presented indirect evidence that the toxin acted at the cell surface. The toxins were not detected inside the fat body tissue, even after long-term exposure enough to cause membranous whorls and disruption of internal structure.

The basal lamina is a heterogeneous layer of polysaccharides reinforced with collagen and elastic fiber (Locke, 1984), so that the toxin may penetrate freely across the layer to the plasma membrane. After arriving at the plasma membrane, toxins might interact with some components of plasma membrane, resulting in the detachment of basal lamina from the plasma membrane.

In fat body tissue, how the toxin interacts with plasma membrane through the basal lamina is not obvious from our results. Basal plasma membrane staining is complicated by the apparent toxin binding to the extracellular basal lamina, and in the case of the midgut, by the light staining of the basal lamina by preimmune antibodies (Ryerse et al., 1990). But the basal lamina of Malpighian tubules show no response to preimmune serum in Ryerse et al.'s study (1990), which is a coincident result with our studies on fat body tissue. In view of studies which demonstrated toxin-induced disruption of basal plasma membranes (Percy and Fast, 1983; Maddrell et al., 1988; Reisner et al., 1989), it would be reasonable to conclude that some of the basal staining is plasma membrane-associated (Ryerse et al., 1990).

Our observations by transmission and scanning electron microscopy suggest that the cytopathic effect is disintegration rather than cell swelling. Only cell contents like lipid granules extrude out from the cell after the lysis of membrane. Whether or not cells swell in response to *B. thuringiensis* toxin may depend on the density of cell cytoskeleton or the composition of culture medium (Reisner et al., 1989). Our results with fat body seem most consistent with a general pore forming cytolytic activity for *B. thuringiensis* toxin. Further investigations are under way to elucidate the binding protein in the plasma membrane of fat body cells.

Acknowledgement

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