Zoological Studies **33**(3): 192-199 (1994)

Histopathological Effects of *Bacillus thuringiensis* δ-endotoxin on the Malpighian Tubules of *Pieris canidia* Larva

Jian-Bin Wang and Wilkin Wai-Kuen Cheung

Department of Biology, Chinese University of Hong Kong, Shatin, N.T., Hong Kong

(Accepted November 25, 1993)

Jian-Bin Wang and Wilkin Wai-Kuen Cheung (1994) Histopathological effects of *Bacillus thuringiensis* δ-endotoxin on the Malpighian tubules of *Pieris canidia* larva. *Zoological Studies* **33**(3): 192-199. The histopathological effects of *Bacillus thuringiensis* subsp. *kurstaki* δ-endotoxin on the Malpighian tubule cells of *Pieris canidia* larva were studied after mucosal and serosal treatment. Results showed that tissues examined after one minute of exposure to the toxin had already undergone fine structural alterations. The number of vacuoles in the cytoplasm increased, and the apical microvilli and the basal intracellular channels became slightly disorganized. The mitochondria, however, appeared normal. After ten minutes of toxin treatment, the ground cytoplasm had large vacuoles or cytoplasmic spaces, the microvilli were damaged extensively, there was extrusion of apical cytoplasm, and the basal infoldings were damaged. After twenty minutes of toxin treatment general cell disintegration was observed. The microvilli were severely damaged, the apical membrane lysed, all the mitochondria became very swollen, the rough endoplasmic reticula and cytoplasm were extremely vacuolated, the basal infoldings were greatly disorganized, and the basement membrane was eroded. These results indicate that *Bacillus thuringiensis* δ-endotoxin could effect cell leakage by forming large pores in cell membranes.

Key words: Bacteria, Insect excretory system, Ultrastructure.

Bacillus thuringiensis subsp. *kurstaki* (Btk) is a widely distributed, spore-forming, gram-positive bacterium (Faust and Bulla 1982, Whiteley and Schnef 1986). It produces a parasporal crystal during the sporulation cycle. When the spore or crystal is ingested by a susceptible insect (primarily lepidopterous larvae) the crystal is activated. This crystal injures the midgut epithelium (Sutter and Raun 1967, Fast and Morrison 1972, Ebersold et al. 1978, Endo and Nishiitsutsuji-Uwo 1980, Ellar et al. 1985, Chiang et al. 1986, Cheung et al. 1990, Cheung and Lam 1993).

The histopathological effects of Btk on Lepidoptera Malpighian tubules have not been reported except in the works of Reisner et al. (1989) on *Calpodes* and in Ryerse et al. (1990) on *Heliothis*. Insect Malpighian tubules possess a one cell thick epithelium generally similar to those of the midgut (Smith 1968, Wigglesworth 1972, Marjota and Ballan-Dufrancais 1984).

Investigating the histopathology of the Malpighian tubule as compared to that of the midgut may help us further understand the mode of action of Btk on insect epithelial membranes in general.

MATERIALS AND METHODS

Preparation of parasporal crystals of *Bacillus thuringiensis* subsp. *kurstaki*

Monoclonal strains of Btk, supplied by the Sandoz Company (U.S.A.), were grown in a modified CHES medium (Chestukina et al. 1980): Casamino acid, 0.5%; yeast extract, 0.4%; glucose, 0.2%; NaCl, 0.5%; $MgSO_4 \cdot 2H_2O$, 0.01%; $CaCl_2 \cdot 2H_20$, 0.05%; pH 7.4. Initially the Btk strain was grown for 7 hours at 28°C and then another 12 hours in a more concentrated form (1% v/v of the preculture). The cultured material was centrifuged at 8,000 rpm and 20°C for 15 min. The pellets collected were resuspended in sterile distilled water and incubated in a shaker at 28°C for 4 days to allow sporulation and autolysis. The mixture of

spores and crystals was then layered onto a 67% urografine solution (Millon and Delcour 1984).

The final solution was centrifuged at 6,000 rpm and 4°C for 2 hours. Btk spores were forced to the bottom of the centrifuge tube. A debris band and a parasporal crystal band were visible (Fig. 1). The crystals were isolated carefully with a Pasteur pipette and resuspended in distilled water (4x); later they were lyophilized and stored at −20°C. The sample contained over 99% pure δ-endotoxin, as reported by Millon and Delcour (1984). Samples of these were viewed with a JEOL JEM-35 scanning electron microscope (Fig. 2).

Solubilization of crystal δ-endotoxin was accomplished by incubating the crystals in 1% (w/v) sodium dodecyl sulfate (SDS), 2% (v/v) δ-mercaptoethanol, 6 M urea, and eqimolar ratio of NaH_2PO_4 and Na_2HPO_4 solution (pH 7.2) for 1 hr at 28°C.

Protein composition of the crystals was determined by the SDS-PAGE procedure (Laemmli 1970). A 3% stacking gel and a 7.5% resolving gel were run on a Mini-protein II electrophoresis cell (Bio-Rad) for 10 min at 50 volts and 1 hr at 100 volts, respectively. Different molecular weights of bovine serum albumins and ovalbumin were used as standards. Traces of 63 kDa toxin and 134 kDa protoxin were easily identifiable (Fig. 3).

Activation of toxins

Five mg of purified crystals (containing the MW 134 and 63 kDa proteins) were suspended in 1 ml of a 1.0% protease (enzyme extract with trypsin, Sigma Company) and 0.2% dithiothreitol in 0.2% CAPS (cyclohexylaminopropane sulfuric acid) buffer , pH 10.5 (Yamamoto and McLaughlin 1981). The solution was incubated at 28°C on a rotary shaker for 24 hr and then assayed against

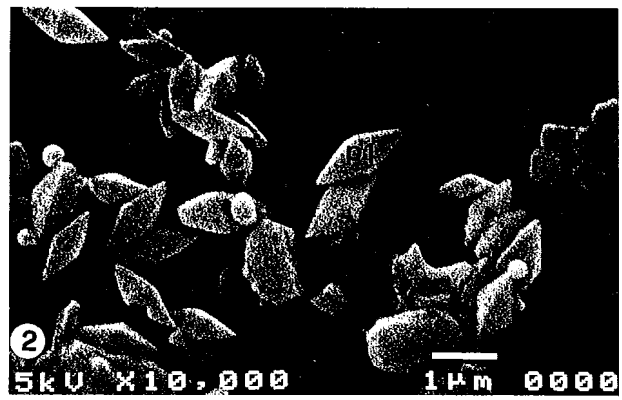


Fig. 2. Scanning electron micrograph of 134 KDa (P1) and traces of 63 KDa (P2) crystals before activation. x12,000.

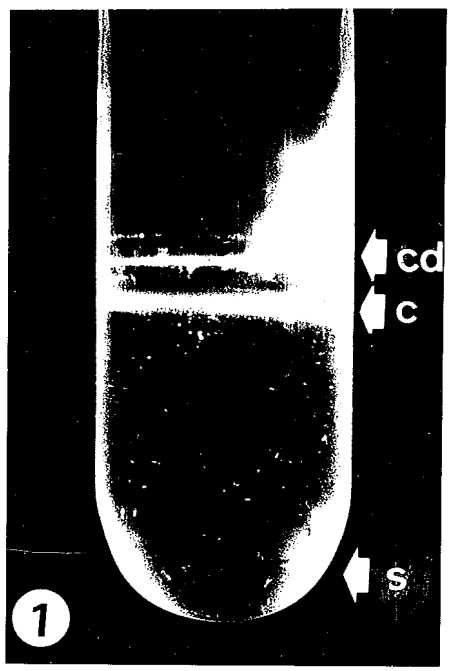


Fig. 1. Separation of spores, crystals, and cell debris of Btk in urografine solution. Shows spores (s), crystal band (c), and cell debris (cd).

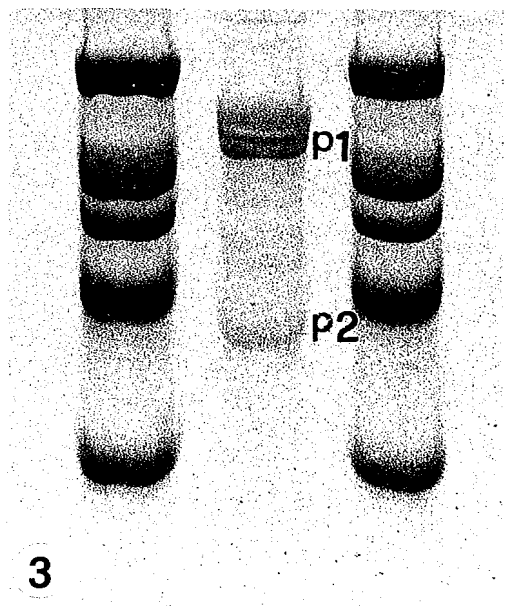


Fig. 3. Solubilized products of Btk crystals (before activation) analyzed by SDS-polyacrylamide gel electrophoresis. Shows 134 KDa protoxin (P1) and traces of 63 KDa toxin (P2). Molecular standards are at both sides.

the earlier mentioned Bio-Rad protein standards. Samples of known concentrations of the 63 kDa toxin were eventually used for treatment of *Pieris canidia* (Talbot 1939) Malpighian tubules. However, the 134 kDa had no noticeable effects on the Malpighian tubules.

Experimental animals and tissue treatment

Larvae of *Pieris canidia* were reared in an insectary according to Cheung et al. (1990). Fourth instar larvae were used for the experiments. For mucosal exposure, 0.00001 μg/ml toxin (in phosphate buffered saline, PBS) was injected into the Malpighian tubule lumen with a micropipette. For serosal exposure Malpighian tubules studied in vivo were immersed in 0.001 μg/ml toxin (in PBS); and those studied in vitro were bent in a U-shape in order just to limit toxin exposure to the arc. Increased toxin concentrations offer results similar to experiments of luminal exposure. The toxin treatments were limited to one, ten, and twenty minute(s), respectively. Controls were performed with buffer only.

Control and toxin treated Malpighian tubules were then fixed in 1% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2, with sucrose). These were post-fixed in 1% osmium tetroxide in 0.2 M phosphate buffer (pH 7.2). Tissues were subsequently blocked in Spurr resin. Thin sections were cut with a Reichart Ultrotome (OMU2 model) and were stained in uranyl acetate and post-stained in lead citrate. Sections were viewed under a Zeiss or JEOL JEM-100C electron microscope.

RESULTS

Malpighian tubule cell structure

Although each *Pieris* Malpighian tubule had regional differentiation in structure and functions (Cheung and Wang 1993) the cellular arrangements appeared principally the same in all four regions i.e. there were approximately one dozen principal cells making up the tubule circumference with only sporadic occurrence of stellate cells. Each principal cell had: 1) a large centrally placed nucleus, 2) numerous basal membranous infoldings forming intracellular channels extending deeply towards the cell apex, and 3) many apical microvilli with mitochondria extending inside them. Most histopathological studies have been carried out on the principal cells of the yellow region since it performs both secretion and mineral storage functions and is the longest of the four regions.

Histopathological effects

One minute post-treatment

Since the Btk endotoxin can bind to membrane receptors (Knowles and Ellar 1987) an exposure of 0.00001 μg/ml of the toxin to the mucosal surface of principal cells for one minute effected damage. The apical microvilli showed slight swelling and the number of vacuoles in the cytoplasm increased (Fig. 4). The mitochondria suffered no notable damage. The nucleus had chromatin materials dispersed and the rough endoplasmic reticula swelled. Basally, the intracellular channels were rather disorganised, but the mitochondria appeared normal (Fig. 5). The basement membrane (0.4 μ thick) was apparently unaffected.

Serosal exposure of principal cells (Figs. 6, 7) with the same dosage of endotoxin did not show any damaging effect. The dosage had to be increased hundred-fold i.e. 0.001 μg/ml before any damage could be noticed. With such dosage the basal channels were disorganised and there were numerous vacuoles in the ground cytoplasm. The apical microvilli also began to show swellings (Fig. 6). The basement membrane remained intact.

Ten minutes post-treatment

Mucosal exposure of the yellow region principal cells showed extensive microvilli damage and there was apical cytoplasm extrusion (Figs. 8, 9). The mitochondria cristae had been modified when compared to the control. The ground cytoplasm had large vacuoles or disorganised cytoplasmic spaces. Apparently the rough endoplasmic reticula had been seriously damaged (Figs. 8, 9). The basal intracellular channels enlarged drastically.

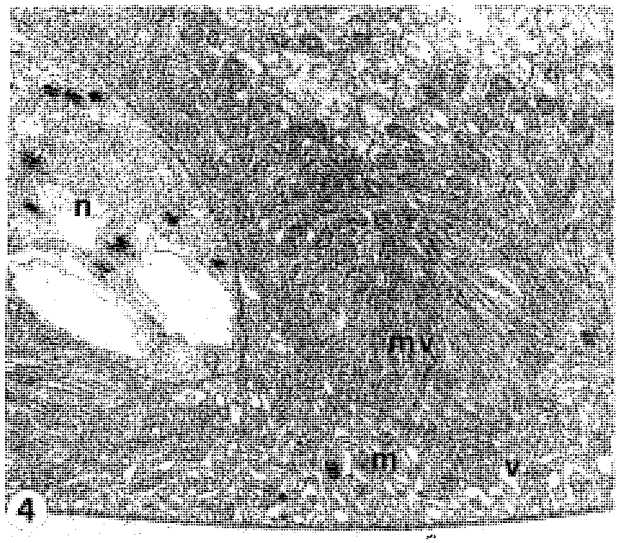
Serosal treatment of the cells resulted in the basal membranous channels becoming disorganised resulting in large vacuoles in the ground cytoplasm (Figs. 10, 11). The mitochondria cristae were seriously damaged. The apical mitochondria appeared to show less damage in their cristae. The whole mitochondrion became round in shape and apparently contracted in size. The microvilli also showed damage (Fig. 10).

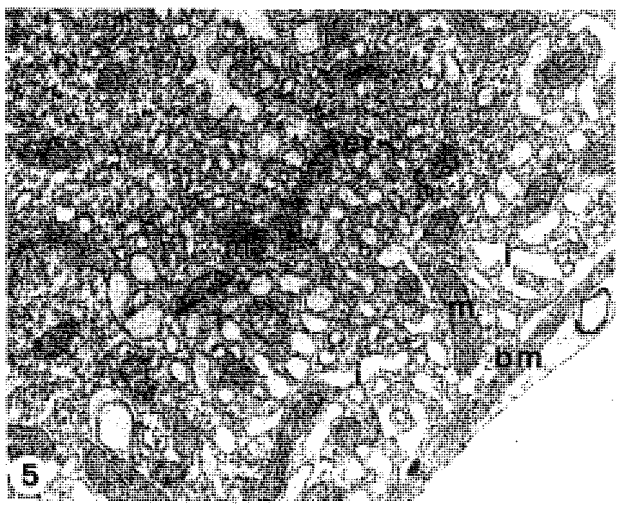
Twenty minutes post-treatment

Mucosal exposure of toxin showed complete damage of apical microvilli (Figs. 12, 13). The mitochondria cristae disintegrated while the

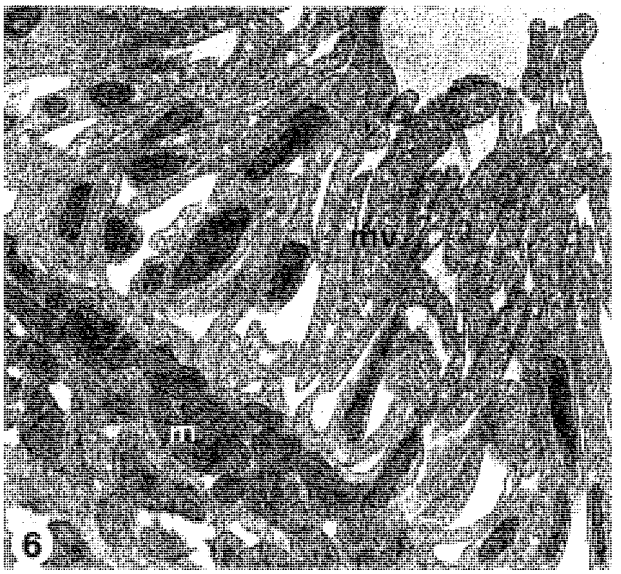
mitochondria themselves became very swollen. The cytoplasm had vacuolated to such an extent that no visible rough endoplasmic reticula could be recognised. Basally the mitochondria became bulbous in shape (Fig. 12) and the basal intracellular channels had suffered much damage. The chromatin materials in the nucleus had all been lost or dispersed. The nucleus had only the nuclear membrane left, resembling almost like an empty bag. The basement membrane, however, could still be recognised.

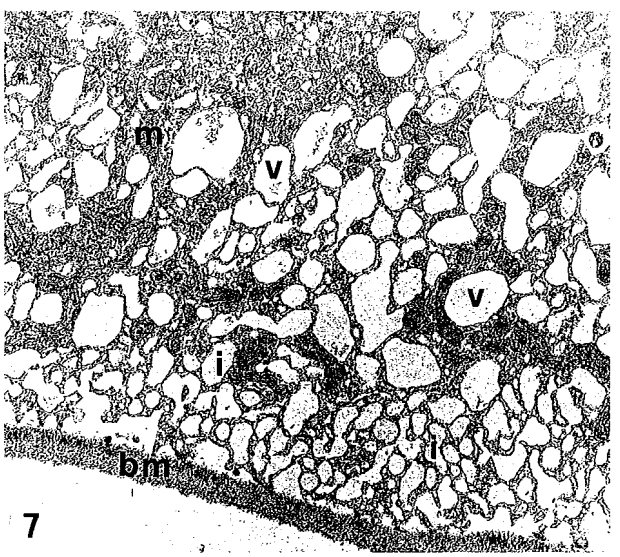
Serosal treatment of the Malpighian tubule cells showed similar damage to that of the mucosal exposure (Figs. 14, 15). The basement membrane had been eroded to some extent. There was serious destruction of the basal intracellular channels. All the mitochondria had their cristae impaired and they became very much swollen. The cytoplasm was filled with large vacuoles. The apical microvilli had been completely destroyed.





Figs. 4-5. 4. Mucosal exposure of principal cell treated with 0.00001 μg/ml toxin for 1 min. Shows numerous vacuoles (v), microvilli (mv), mitochondria (m), and the nucleus (n) with dispersed chromatin materials. x4,300. 5. As above. Basal region. Shows mitochondria (m), basement membrane (bm), rough endoplasmic reticulum (er), and disorganised intracellular channels (i). x6,600.



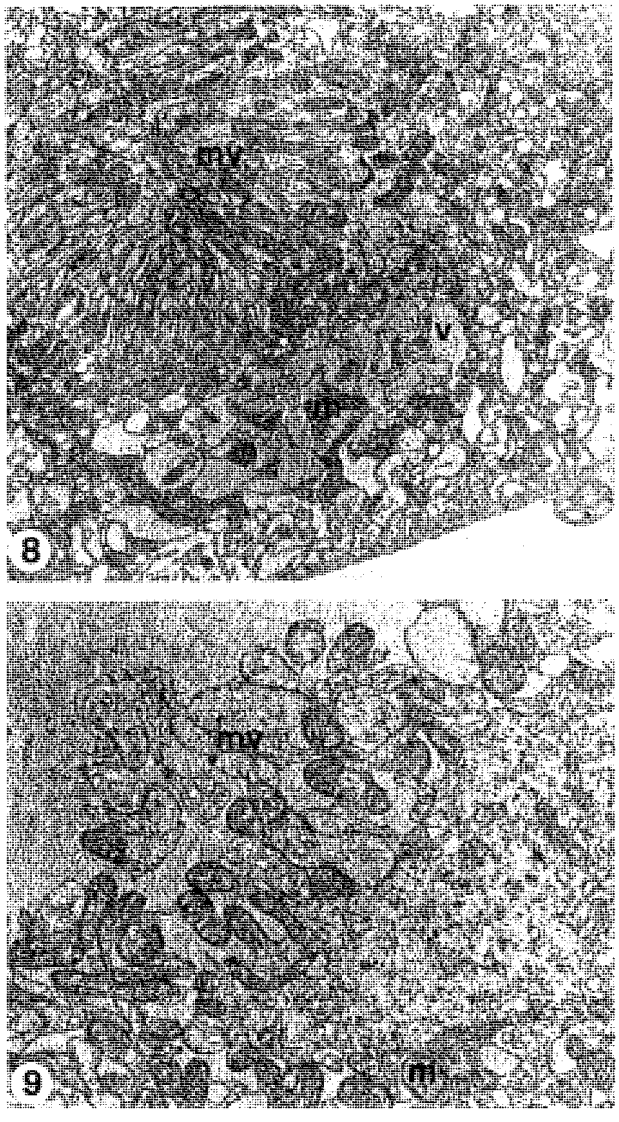


Figs. 6-7. 6. Serosal exposure of principal cell treated with 0.001 μg/ml toxin for 1 min. Shows relatively undamaged microvilli (mv) and normal mitochondria (m). x7,600. 7. As above. Basal region. Shows disorganised intracellular channels (i), numerous vacuoles (v), and mitochondria (m). x7,600.

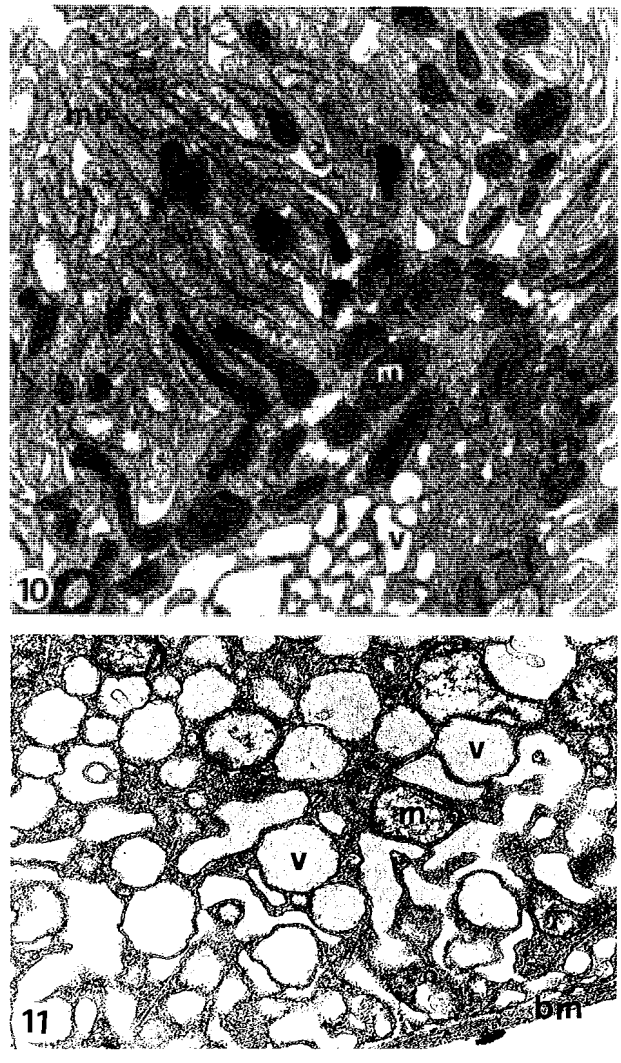
DISCUSSION

Heimpel and Angus (1959) reported that the action of Bt on different lepidopterous larvae (primarily acting on the midgut) differed in their response. There were caterpillars which showed immediate response to Bt treatment (within one to seven hours). There were caterpillars which died within two to seven days and finally there were caterpillars which were killed by septicemia rather than the toxin.

Reisner et al. (1989) studied the histopathological effects of Btk δ-endotoxin on *Calpodes* Malpighian tubules. At one hour post-treatment, the principal cells exhibited various cytopathological changes with a progression of severity from low to high toxin concentration. Mucosal exposure of cells to 16 μg/ml 63 kDa toxin completely inhibited urine secretion and induced massive cytolysis. Serosal exposure required a 20-fold higher concentration (314 μg/ml toxin) to bring about a similar effect. Mucosal exposure resulted first in microvilli



Figs. 8-9. 8. Mucosal exposure of principal cell treated with 0.00001 μg/ml toxin for 10 min. Shows damaged microvilli (mv), numerous vacuoles (v), and mitochondria (m). x4,600. 9. As above. Apical region. Shows damaged microvilli (mv) and mitochondria (m). x7,600.



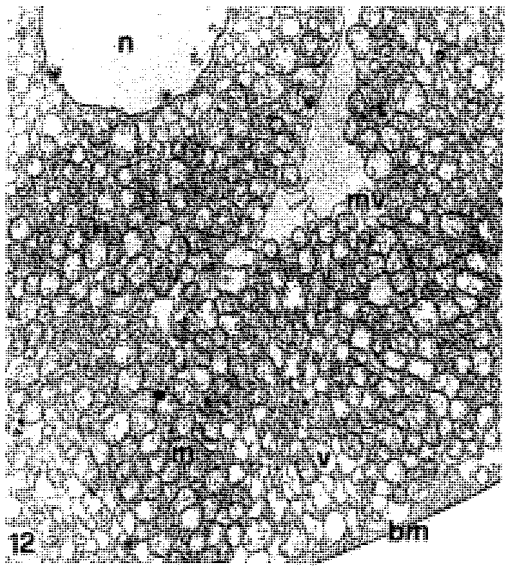
Figs. 10-11. 10. Serosal exposure of principal cell treated with 0.001 μg/ml toxin for 10 min. Shows microvilli (mv), vacuoles (v), and mitochondria (m). x7,400. 11. As above. Basal region. Shows numerous vacuoles (v), mitochondria (m) with cristae damaged, and basement membrane (bm). x8,700.

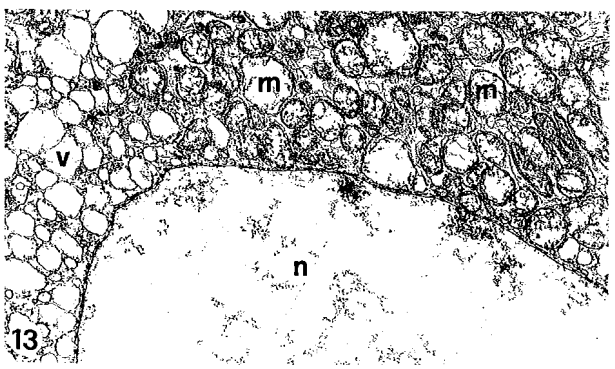
disruption whilst serosal exposure resulted in damage to the basal infolds first. The progress from low to severe damage could be broadly categorized into 3 stages: stage 1 had only slight enlargement of cytoplasmic spaces, stage 2 had further enlargement of these spaces and stage 3 had complete destruction of microvilli, basal infolds, endoplasmic reticula, and mitochondria.

Our results on *Pieris* larvae showed similar effects though the required toxin concentration was much lower and the time required to have comparable cytopathological damages was much shorter. This might indicate that *Pieris* larvae could be more susceptible to Btk δ-endotoxin impairment than *Calpodes* larvae.

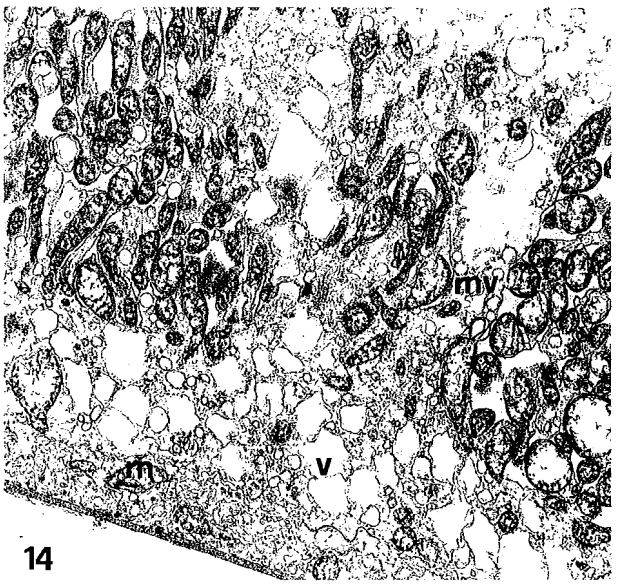
Pieris Malpighian tubules apparently show a more sensitive response than its midgut cells to Btk treatment. Cheung et al. (1990) found that *Pieris* midgut cells required at least one hour of exposure in order to have similar organelle damage. The result discrepancies might be due to the fact that midgut cells were larger in size and the Btk toxin used in the midgut experiment was a crude spore-toxin mixture instead of purified δ-endotoxin.

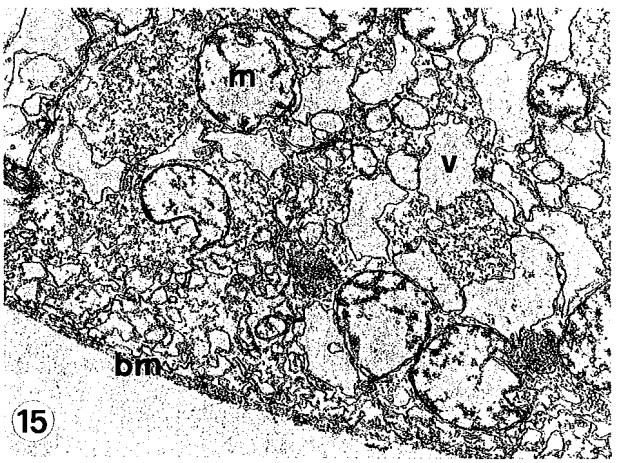
When purified δ-endotoxin was used in experiments on other lepidopterous larvae, other toxicologists were able to demonstrate that the Btk δ-endotoxin also acts quickly on midgut cells. For example, Percy and Fast (1983) noticed that





Figs. 12-13. 12. Mucosal exposure of principal cell treated with 0.00001 μg/ml toxin for 20 min. Shows damaged microvilli (mv), nucleus (n), mitochondria (m), vacuoles (v), and basement membrane (bm). x3,100. 13. As above. Part of nuclear region. Shows numerous vacuoles (v), damaged mitochondria (m), and nucleus (n) with chromatin materials lost. x4,600





Figs. 14-15. 14. Serosal exposure of principal cell treated with 0.001 μg/ml toxin for 20 min. Shows damaged microvilli (mv), vacuoles (v), and mitochondria (m). x4,200. 15. As above. Basal region. Shows damaged basement membrane (bm), numerous vacuoles (v), and mitochondria (m) with cristae impaired. x8,500.

the *Bombyx* midgut cells exhibited cytopathological effects after a one minute treatment. However, slightly longer times were required to have similar results for *Manduca* (Gupta et al. 1985, Lane et al. 1989).

As pointed out by Reisner et al. (1989), response differences could be due to a variety of variables associated with the tissue type, time, and concentration of toxin exposure. Other factors such as the developmental stage and fixation conditions might add to the complications.

In general, it might be said that the primary effect of Btk δ-endotoxin is cellular membranes damage. Several hypotheses have been proposed to explain the mechanism of the δ-endotoxin: 1) it binds to a specific plasma membrane and forms lytic pores (Knowles and Ellar 1987), 2) it uncouples oxidative phosphorylation (Travers et al. 1976), 3) it specifically affects Na^+ and/or K^+ transport (Himeno et al. 1985), 4) it inhibits an epithelial K^+ pump (Gupta et al. 1985), 5) it causes a general breakdown of cell permeability barriers (Nishiitsitsuji-Uwo and Endo 1980).

Our results showed that Bt δ-endotoxin had damaging effects on epithelial membranes other than the midgut. It might create lytic pores by binding to specific membrane areas or receptors resulting in osmotic lysis of cells as proposed by Knowles and Ellar (1987).

Ryerse et al. (1990) demonstrated the presence of Bt binding areas on the midgut and Malpighian tubule membranes of *Heliothis* with an immunofluoresent method at the light microscopic level. These "receptors" were randomly distributed all over the exposed epithelial membranes. Thus future specific antibody probes at the ultrastructural level need to be carried out in order to better understand the nature and distribution of these receptors.

REFERENCES

Chestukhina GG, IA Zalunin, LI Kostina, TS Kotova, SP Kattruka, VM Stepanov. 1980. Crystal-forming proteins of *Bacillus thuringiensis*. Biochem. J. **187:** 457-465.

Cheung WWK, YF Lam. 1993. Histochemical studies on normal and *Bacillus thuringiensis*-infected *Pieris canidia* larval midgut. Bull. Inst. Zool., Acad. Sinica **32(1):** 12-22.

Cheung WWK, YF Lam, ST Chang. 1990. Histopathological effects of *Bacillus thuringiensis* on *Pieris canidia* larva: A transmission electron microscopic study. Acta Biol. Exp. Sinica **23:** 177-191.

Cheung WWK, JB Wang. 1993. Ultrastructural differentiation of the Malpighian tubules of *Pieris canidia* Spar. larva. Acta Biol. Exp. Sinica **26:** 197-213.

Chiang AS, DF Yen, WK Peng. 1986. Defense reaction of midgut epithelial cells in rice moth larva *Corcyra cephalonica* infected with *Bacillus thuringiensis*. J. Invertebr. Pathol. **47:** 333-339.

Ebersold HR, P Luthy, P Geiser, L Ettlinger. 1978. The action of the δ-endotoxin of *Bacillus thuringiensis*: An electron microscopic study. Experientia **34:** 1672.

Ellar DJ, WE Thomas, BH Knowles, S Ward, J Todd, F Brobniewski, J Lewis, T Sawyer, D Last, C Nichols. 1985. Biochemistry, genetics and mode of action of *Bacillus thuringiensis* δ-endotoxins. *In* Molecular Biology of Microbial Differentiation, eds. JA Hoch, P Setlow. Washington: American Society of Microbiology, pp. 230-240.

Endo Y, J Nishiitsutsuji-Uwo. 1980. Mode of action of *Bacillus thuringiensis* δ-endotoxin: Histopathological changes in the silkworm midgut. J. Invertebr. Pathol. **36:** 90-103.

Fast PG, IK Morrison. 1972. The δ-endotoxin of *Bacillus thuringiensis*. IV. The effect of δ-endotoxin on ion regulation by midgut tissue of *Bombyx mori* larvae. J. Invertebr. Pathol. **20:** 208-211.

Faust RM, LA Bulla Jr. 1982. Bacteria and their toxins as insecticides. *In* Microbial and Viral Pesticides, ed. E Kurstak. New York: Marcel Dekker, pp. 75-208.

Gupta BL, JAT Dow, TA Hall, WR Harvey. 1985. Electron probe x-ray microanalysis of the effects of *Bt* var. *kurstaki* crystal protein insecticide on ions in an electronic K^+-transporting epithelium of the larval midgut in the Lepidopteran, *Manduca sexta*, *in vitro*. J. Cell Sci. **74:** 137-152.

Heimpel AM, TA Angus. 1959. The site of action of crystalliferous bacteria in Lepidoptera larvae. J. Insect Pathol. **1:** 152-170.

Himeno MK, N Koyama, T Funato, T Komano. 1985. Mechanism of action of *Bacillus thuringiensis* insecticidal delta-endotoxin on insect cells *in vitro*. Agric. Biol. Chem. **49:** 1461-1468.

Knowles BH, DJ Ellar. 1987. Colloid-osmotic lysis is a general feature of the mechanism of action of *Bacillus thuringiensis* δ-endotoxins with different insect specificity. Biochim. Biophys. Acta **924:** 509-518.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680-685.

Lane NJ, JB Harrison, WM Lee. 1989. Changes in microvilli and golgi-associated membranes of Lepidopteran cells induced by an insecticidally active bacterial δ-endotoxin. J. Cell Sci. **93:** 337-347.

Martoja R, C Ballan-Dufrancais. 1984. The ultrastructure of digestive and excretory organs. *In* Insect Ultrastructure, Vol. 2, eds. RC King, H Akai. New York: Academic Press, pp. 199-268.

Millon J, J Delcour. 1984. A convenient procedure for the preparation of highly purified parasporal crystals of *Bacillus thuringiensis*. J. Microbiol. Methods **3:** 69-76.

Nishiitsutsuji-Uwo J, Y Endo. 1980. Mode of action of *Bacillus thuringiensis* δ-endotoxin: General characteristics of intoxicated *Bombyx* larvae. J. Invertebr. Pathol. **35:** 219-228.

Percy J, PG Fast. 1983. *Bacillus thuringiensis* crystal toxin: Ultrastructural studies of its effect on silkworm midgut cells. J. Invertebr. Pathol. **41:** 86-98.

Reisner WM, DJ Feir, PB Lavrik, JS Ryerse. 1989. Effect of *Bacillus thuringiensis kurstaki* δ-endotoxin on insect Malpighian tubule structure and function. J. Invertebr. Pathol. **54:** 175-190.

Ryerse JS, JR Beck Jr., PB Lavrik. 1990. Light microscopic immunolocation of *Bacillus thuringiensis kurstaki* δ-endotoxin in the midgut and Malpighian tubules of the tobacco budworm, *Heliothis virescens*. J. Invertebr. Pathol. **56:** 86-90.

Smith DS. 1968. Insect cells: Their structure and functions. Edinburgh: Oliver & Boyd.

Sutter GR, SS Raun. 1967. Histopathology of European corn borer larvae treated with *Bacillus thuringiensis*. J. Invertebr. Pathol. **9:** 90-103.

Talbot G. 1939. Fauna of British India. London: Taylor and Francis.

Travers RS, RM Faust, CF Reichelderfer. 1976. Effects of *Bacillus thuringiensis* var. *kurstaki* δ-endotoxin on isolate Lepidopteran mitochondria. J. Invertebr. Pathol. **28:** 351-356.

Whiteley HR, HE Schnepf. 1986. The molecular biology of parasporal crystal body formation in *Bacillus thuringiensis*. Ann. Rev. Microbiol. **40:** 549-576.

Wigglesworth VB. 1972. The principles of insect physiology. 7th ed. London: Chapman and Hall.

Yamamoto T, RE McLaughlin. 1981. Isolation of a protein from the parasporal crystal of *Bacillus thuringiensis* var. *kurstaki* toxic to the mosquito larva, *Aedes taeniorhychus*. Biochem. Biophy. Res. Comm. **103:** 414-421.

蘇力菌δ－內毒素對東方粉蝶幼蟲馬氏管之組織病理影響

王 建 斌[1] 張 偉 權[1]

東方粉蝶幼蟲馬氏管細胞經蘇力菌δ－內毒素在粘膜面和漿膜面處理後，對其組織病理影響進行研究。結果顯示經過毒素一分鐘處理後的組織，其超微結構已經改變。這些變化包括細胞質中液泡增加，頂部微絨毛和基部細胞膜內管道輕微紊亂，但線粒體仍然顯示正常。經毒素10分鐘處理後，細胞質有些大液泡或大細胞間隙，微絨毛受到廣泛的破壞，並且頂部細胞質從細胞中擠出，同時基部細胞膜內折疊也受到損壞。經毒素20分鐘處理後，可觀察到整個細胞已經分解，微絨毛完全損壞，頂膜溶解，所有線粒體都變成了膨脹形，粗糙內質網和細胞質極大地液泡化，基部細胞膜內折疊十分紊亂，並基膜損壞。這些結果表明蘇力菌δ－內毒素能通過和細胞膜結合，引至產生大孔，導致細胞漏泄。

關鍵詞：細菌，昆蟲排泄系統，超微構造。

[1] 香港中文大學生物系