

## BACILLUS THURINGIENSIS VAR ISRAELENسيس CRYSTAL $\delta$ -ENDOTOXIN: EFFECTS ON INSECT AND MAMMALIAN CELLS *IN VITRO* AND *IN VIVO*

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### SUMMARY

*Bacillus thuringiensis var israelensis* parasporal crystal  $\delta$ -endotoxin was purified by ultracentrifugation on a discontinuous sucrose gradient. Native  $\delta$ -endotoxin crystals showed no detectable toxicity in the *in vitro* and *in vivo* systems that are described. By contrast alkali-solubilized crystal  $\delta$ -endotoxin caused rapid cytological and cytopathological changes in *Aedes albopictus*, *Choristoneura fumiferana* 63 CF1, *Spodoptera frugiperda* and *Trichoplusia ni* cell lines as observed by phase-contrast microscopy and vital staining.

Mouse fibroblasts, primary pig lymphocytes and three mouse epithelial carcinoma cell types showed a similar response to the alkali-soluble crystal  $\delta$ -endotoxin. In addition the soluble crystal  $\delta$ -endotoxin protein caused haemolysis of rat, mouse, sheep, horse and human erythrocytes.

Intravenous administration of the alkali-soluble crystal  $\delta$ -endotoxin to Balb.c mice at a dose rate of 15–30  $\mu$ g of protein per gram body weight resulted in rapid paralysis followed by death within 12 h. Subcutaneous inoculation of 15–30  $\mu$ g of protein per gram body weight resulted in death of suckling mice in 2–3 h. The alkali-solubilized crystal  $\delta$ -endotoxin was not toxic however, when administered per os.

A comparison is made with a similar alkali-soluble fraction from the parasporal crystal  $\delta$ -endotoxin of *B. thuringiensis var kurstaki*. With the exception of the Lepidopteran cell line, *Choristoneura fumiferana* 63 CF1, this soluble crystal  $\delta$ -endotoxin protein showed no *in vitro* or *in vivo* toxicity, and no haemolytic activity.

### INTRODUCTION

During the process of sporulation of the gram-positive bacterium *Bacillus thuringiensis* a parasporal, proteinaceous, crystalline inclusion is synthesized within the sporangium (Somerville, 1978; Bulla *et al.* 1980). The insecticidal properties of this protein crystal  $\delta$ -endotoxin have stimulated intensive study over the past 30 years leading to the commercial production of the  $\delta$ -endotoxin and its widespread use as a biological control agent. Based on the antigenic properties of their flagellar H-antigens, 19 varieties of *B. thuringiensis*, belonging to 14 serotypes, have been identified. Thirteen of the serotypes are active against more than 182 species of insect, in particular against pest species of the order Lepidoptera. The fourteenth serotype, *B. thuringiensis var israelensis* (Goldberg & Margalitt, 1977) is of potential importance since, unlike the other serotypes, it is highly toxic for certain Diptera; notably, mosquitos and blackfly (de Barjac, 1978a). The prospect of employing the crystal  $\delta$ -endotoxin from this serotype as a biological control agent against these insects, which present a major third-world health problem, has stimulated considerable

interest in characterizing the *B. thuringiensis var israelensis* crystal toxin and discovering the molecular basis of its mode of action.

The biochemistry of *B. thuringiensis var israelensis* crystal  $\delta$ -endotoxin has been investigated and compared with that of the crystal from other *B. thuringiensis* serotypes (Tyrell *et al.* 1981).

Lepidopteran larvae when fed *B. thuringiensis* (serotypes 1, 3a, 4a, 4b, 6, 7, 8, 9) show rapid gut paralysis, sluggish behaviour, cessation of feeding and finally death (Heimpel & Angus, 1960; Cooksey, 1968). Histological studies (Angus, 1970; Ebersold, Lüthy, Geiser & Ettliger, 1978; Endo & Nishiitsutsuji-Uwo, 1980) have indicated that the toxin causes gross disruption of the midgut epithelium; cells balloon and lyse with severe disruption of the gut wall. Similarities have been observed between this typical pathology and the symptoms induced by the parasporal crystal  $\delta$ -endotoxin of *B. thuringiensis var israelensis*. de Barjac observed that ng amounts of *israelensis* crystal  $\delta$ -endotoxin caused cessation of movement and subsequent death of *Aedes aegypti* larvae (de Barjac, 1978a). de Barjac also presented histopathological evidence that the gut epithelium is the primary target; the gut epithelium showed the typical swelling followed by general tissue lysis (de Barjac, 1978b).

*In vitro* tissue culture of insect cell lines has previously been used in the investigation of the mode of action of *B. thuringiensis* serotypes active against Lepidoptera. It was found that cultured insect cells from three types of Lepidoptera responded to enzyme-digested parasporal crystal  $\delta$ -endotoxin with swelling, vesicle formation and lysis (Murphy, Sohi & Fast, 1976; Geiser, 1979). Similar effects were observed with the Lepidopteran TN-368 cell line (Nishiitsutsuji-Uwo, Endo & Himeno, 1979). As yet there are no reports of the *in vitro* activity of *B. thuringiensis var israelensis* in insect cell lines. This paper reports the cytological effects of an alkali-solubilized fraction from the *israelensis* parasporal crystals on several insect and mammalian cell lines. The results of intravenous and subcutaneous inoculation and oral feeding of the alkali-solubilized crystal  $\delta$ -endotoxin to Balb.c mice are also described. Results with the *israelensis* crystal  $\delta$ -endotoxin are compared with the results of parallel experiments using an alkali soluble fraction from the crystal  $\delta$ -endotoxin of *B. thuringiensis var kurstaki*.

## MATERIALS AND METHODS

### *Microorganisms and growth conditions*

*B. thuringiensis var israelensis* (IPS 78) was obtained from Professor H. de Barjac (Institut Pasteur, Paris). *B. thuringiensis var kurstaki* (HDI) was obtained from Dr H. D. Burges (Glasshouse Crops Research Institute, England).

Growth and sporulation of both serotypes were as described for *Bacillus megaterium* KM (Stewart, Johnstone, Hagelberg & Ellar, 1981).

### *Purification of B. thuringiensis var israelensis crystal $\delta$ -endotoxin*

Spores and crystals were harvested by transferring the culture into 500 ml centrifuge bottles on ice and pelleting the spores and crystals at 6000–7000 *g* for 5–10 min at 4 °C. After centrifugation, sporangial debris was removed from the pellet by washing with ice-cold deionized water. This was

repeated up to six times until spores and crystals were free from debris when viewed by phase-contrast microscopy. The final pellet was resuspended in 50 mM-Tris·HCl (pH 7·5). Spores and crystals were separated using differential ultracentrifugation through a discontinuous sucrose density gradient. A spore/crystal mixture (50 mg) was layered on top of a 30 ml discontinuous sucrose gradient, comprising 10 ml each of 67%, 72% and 79% (w/v) sucrose in 50 mM-Tris·HCl (pH 7·5) containing 10 mM-KCl. Centrifugation was carried out in a Beckman L5-50 ultracentrifuge in a SW25·1 rotor operating at 80 000 *g* for 14 h at 4°C. Crystals formed a major band at the interface between the 72% and 79% (w/v) sucrose, while the spores formed a discrete pellet at the bottom of the tube. The crystal band was removed and washed three times in ice-cold 50 mM-Tris·HCl (pH 7·5) by centrifugation at 15 000 *g* for 5 min at 4°C, to remove the sucrose. The final pellet was resuspended in deionized water and stored frozen at -20°C. Purity of the crystal  $\delta$ -endotoxin preparations was monitored by phase-contrast microscopy and by the production of bacterial colonies on CCY agar plates (Stewart *et al.* 1981) by serial dilution of crystal preparations.

#### *Purification of B. thuringiensis var kurstaki crystal $\delta$ -endotoxin*

The method was the same as that for *var israelensis* except for the following: after harvesting, the final spore/crystal pellet was resuspended in deionized water. Spores and crystals were separated on a discontinuous sucrose gradient of 45%, 67% and 87% (w/v) sucrose in deionized water. The crystals formed a major band at the interface of the 67% and 87% (w/v) sucrose. These were removed from the gradient and washed in ice-cold deionized water to remove sucrose.

#### *Protein estimation*

Protein concentration was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

#### *Electrophoresis*

Sodium dodecyl sulphate (SDS)/polyacrylamide gel electrophoresis was conducted as described by Laemmli & Favre (1973), an acrylamide (Fisons):*N,N'*-methylenebisacrylamide (BDH) ratio of 100:1 being used. Protein samples were completely solubilized by incubation for 5 min at 100°C in SDS/sample buffer (50 mM-Tris·HCl (pH 7·5), 1% (w/v) SDS, 25 mM-dithiothreitol, 2 mM-phenylmethanesulphonyl fluoride, 1 mM-ethylenediaminetetraacetic acid, 10% (w/v) glycerol, 0·0025% (w/v) Bromophenol Blue). Samples were applied to a 13% gel run at 20 mA constant current. The gel was stained overnight at room temperature with 0·1% (w/v) Coomassie Brilliant Blue R in 50% (v/v) methanol, 10% (v/v) acetic acid and destained with several changes of 10% (v/v) propan-2-ol, 10% (v/v) acetic acid. Molecular weights were estimated by the relation of  $\log M_r$  to the  $R_F$  of Sigma SDS/polyacrylamide gel electrophoresis marker proteins.

#### *Solubilization of B. thuringiensis var israelensis crystal $\delta$ -endotoxin*

Crystal  $\delta$ -endotoxin at 2 mg/ml was treated with 50 mM- $\text{Na}_2\text{CO}_3\cdot\text{HCl}$  (pH 10·5) by incubation at 37°C for 60 min. Insoluble material remaining was removed by centrifugation at 10 000 *g* for 10 min and the supernatant containing soluble  $\delta$ -endotoxin material was removed for subsequent bioassay. The pellet was resuspended in phosphate-buffered saline (pH 7·0). Protein estimations were carried out on each fraction.

#### *Solubilization of B. thuringiensis var kurstaki crystal $\delta$ -endotoxin*

Crystal  $\delta$ -endotoxin was solubilized by the method of Huber, Lüthy, Ebersold & Cordier (1981).

#### *Cells*

*Aedes albopictus* (Dipteran, mosquito adult ovarian tissue) were grown in Mitsuhashi Maramorosch medium (GIBCO) containing 10% foetal calf serum (GIBCO). *Choristoneura fumiferana* 63 CFl (Lepidopteran, spruce budworm, trypsinized larval tissue) obtained from Dr S. S. Sohi (Canadian Forest Pest Management Institute, Ontario) were grown in 86% Graces medium (GIBCO) containing 14% foetal calf serum, and 0·25% (w/v) tryptose broth (DIFCO).

*Spodoptera frugiperda* (Lepidopteran, fall army worm, pupal ovary) and *Trichoplusia ni* (Lepidopteran, cabbage looper adult ovary) were grown in TC 100 medium (Gardiner & Stockdale, 1975), containing 10% foetal calf serum. Mouse fibroblasts (L929) were obtained from Dr A. A. Newton (Department of Biochemistry, Cambridge). Mouse epithelial carcinoma cell lines (EC2, EC5 and EC6) were obtained from Miss E. Robertson (Department of Genetics, Cambridge). Primary pig lymphocytes were obtained from Miss S. Felber (Department of Biochemistry, Cambridge). All media contained 50 µg/ml gentamicin (Nicholas laboratories) and 50 µg/ml fungizone (GIBCO).

### Tissue culture

Cell lines were grown in 25 cm<sup>2</sup> tissue culture flasks (Nunc), or in 250 ml spinner culture flasks on a Belco magnetic stirrer, at 27°C. Confluent monolayers were washed once in 5 ml phosphate-buffered saline (PBS), the test sample was applied and then 2 ml medium without foetal calf serum was added. Results were recorded by photography using phase-contrast microscopy and an Olympus OM2 camera attachment.

Prior to the addition of test samples, *A. albopictus* and *C. fumiferana* 63 CF1 cells grown in 250 ml spinner culture flasks, were decanted, pelleted by low-speed centrifugation and resuspended at a cell concentration of 1–3 ( $\times 10^6$ )/ml in 5 ml of tissue-culture medium without foetal calf serum. At various time intervals after the addition of the test sample, cell viability was measured by vital staining (0.1% (w/v) Trypan Blue (Sigma), 0.002% (v/v) glacial acetic acid in PBS) using a haemocytometer counting chamber.

### Assay for haemolytic activity

Erythrocytes were washed three times in 50% (v/v) PBS, 2.25% (w/v) glucose by low-speed centrifugation for 5 min in a graduated centrifuge tube. The volume of packed cells was noted and a 0.1% (v/v) suspension was made in 50% (v/v) PBS, 0.05% (w/v) gelatin, 2.25% (w/v) glucose.

Twofold serial dilutions of test samples were made in round-well microtitre plates (Linbro) in 100 µl of 50% (v/v) PBS, 0.05% (w/v) gelatin, 2.25% (w/v) glucose. A sample (100 µl) of 0.1% erythrocyte solution was then added to each well. Dilution of the test samples and addition of erythrocytes was carried out either at room temperature or at 4°C. The 4°C plates were left at 4°C for 3 h, after which time they were placed at room temperature. *B. thuringiensis var israelensis* undiluted test samples titrated in horse, human and sheep erythrocytes were: (1) 20 µg of alkali-solubilized crystal  $\delta$ -endotoxin protein (1 µg/µl) in 50 mM-Na<sub>2</sub>CO<sub>3</sub>·HCl (pH 10.5); (2) 20 µg of native crystal  $\delta$ -endotoxin; (3) 20 µg of the insoluble protein remaining after incubation of native crystal  $\delta$ -endotoxin with 50 mM-Na<sub>2</sub>CO<sub>3</sub>·HCl (pH 10.5); and (4) 20 µl of 50 mM-Na<sub>2</sub>CO<sub>3</sub>·HCl (pH 10.5). *B. thuringiensis var kurstaki* undiluted test samples titrated in horse and human erythrocytes were: (1) 50 µg of alkali-solubilized crystal  $\delta$ -endotoxin protein (Huber *et al.* 1981), precipitated with saturated ammonium sulphate and resuspended in 50 µl of 50 mM-Na<sub>2</sub>CO<sub>3</sub>·HCl (pH 9.5); (2) 50 µg of native crystal  $\delta$ -endotoxin; (3) 50 µg of the insoluble protein remaining after solubilization of the native crystal  $\delta$ -endotoxin in 50 mM-Na<sub>2</sub>CO<sub>3</sub>·HCl (pH 9.5), 10 mM-dithiothreitol; and (4) 50 µl of 50 mM-Na<sub>2</sub>CO<sub>3</sub>·HCl (pH 9.5).

All test samples were made up to a final volume of 100 µl with PBS prior to serial dilution in the microtitre plates.

### *B. megaterium* KM protoplast preparation

*B. megaterium* KM protoplasts were prepared as described by Bernheimer & Schwartz (1965).

### In vivo assay

*In vivo* toxicity of the *B. thuringiensis* serotypes for adult Balb.c mice (30 g) was tested by administration either intravenously by tail vein inoculation, or by oral feeding, of: (1) 0.1 mg, 0.5 mg and 1.0 mg of solubilized crystal  $\delta$ -endotoxin, concentrated by precipitation with saturated ammonium sulphate on ice for 1 h (the precipitates were washed and resuspended in 0.2–0.4 ml of 50 mM-Na<sub>2</sub>CO<sub>3</sub>·HCl (pH 10.5)); (2) 0.5 mg and 1.0 mg of native crystal  $\delta$ -endotoxin in 0.4 ml PBS;

and (3) an equivalent volume of 50 mM- $\text{Na}_2\text{CO}_3 \cdot \text{HCl}$  (pH 10.5). Toxicity of lower concentrations of these samples was also tested by subcutaneous inoculation in 1- to 2-week-old suckling Balb.c mice.

## RESULTS

The crystalline  $\delta$ -endotoxin from both serotypes of *B. thuringiensis* was conveniently separated from spores and purified by differential ultracentrifugation on discontinuous sucrose density gradients as described in Materials and Methods. Residual spore contamination in the purified crystal  $\delta$ -endotoxin was determined as  $\leq 0.2\%$  by viable colony counts on CCY agar.

### *B. thuringiensis* var *israelensis*

Bioassay of the purified crystal  $\delta$ -endotoxin in *A. aegypti* 3rd instar larvae (Tyrell, Davidson, Bulla & Ramoska, 1979) gave an  $\text{LC}_{50}$  (concentration that will be lethal to 50% of the larvae) of 5–50 ng protein/ml.

Total solubilization of the crystal  $\delta$ -endotoxin was achieved by incubation at 100 °C for 5 min in SDS/sample buffer. The polypeptide profile of the crystal  $\delta$ -endotoxin analysed by SDS/polyacrylamide gel electrophoresis is shown in Fig. 1, track 4. The major *israelensis* polypeptide had a  $M_r$  of 28 000 in agreement with previous reports (Tyrell *et al.* 1981).

Incubation of the crystal  $\delta$ -endotoxin with 50 mM- $\text{Na}_2\text{CO}_3 \cdot \text{HCl}$  (pH 10.5) for 30 min at 37 °C resulted in the partial solubilization of several crystal components (Fig. 1, track 5) including the 28 000  $M_r$  polypeptide. These alkali-soluble polypeptides represented 40% of the total crystal protein. The insoluble protein recovered after centrifugation is represented by Fig. 1, track 6. It is of particular interest that the two high molecular weight polypeptides ( $M_r$  98 000 and 93 500) are recovered exclusively in the pellet.

### *B. thuringiensis* var *kurstaki*

Bioassay of the purified crystal  $\delta$ -endotoxin in *Pieris brassicae* 3rd instar larvae (Cooksey, 1968) gave an  $\text{LC}_{50}$  of 10–100 ng protein/ml.

Complete solubilization of the crystal  $\delta$ -endotoxin was achieved by incubation at 100 °C for 5 min in SDS/sample buffer. Analysis of the total polypeptide profile by SDS/polyacrylamide gel electrophoresis is illustrated in Fig. 1, track 3. The major polypeptide components have  $M_r$  values of 126 000 and 63 000. Fig. 1, tracks 1 and 2 represent the soluble and insoluble crystal  $\delta$ -endotoxin protein obtained after incubation of the native crystal with 50 mM- $\text{Na}_2\text{CO}_3 \cdot \text{HCl}$  (pH 9.5), 10 mM-dithiothreitol for 60 min at 37 °C (Huber *et al.* 1981). The 126 000  $M_r$  polypeptide was found almost exclusively in the soluble fraction while the majority of the 63 000  $M_r$  polypeptide remained insoluble. The soluble polypeptides represented 90% of the total crystal protein.

In contrast to *B. thuringiensis* var *israelensis*, *B. thuringiensis* var *kurstaki* was insoluble in the alkaline buffer in the absence of dithiothreitol. It should be noted that

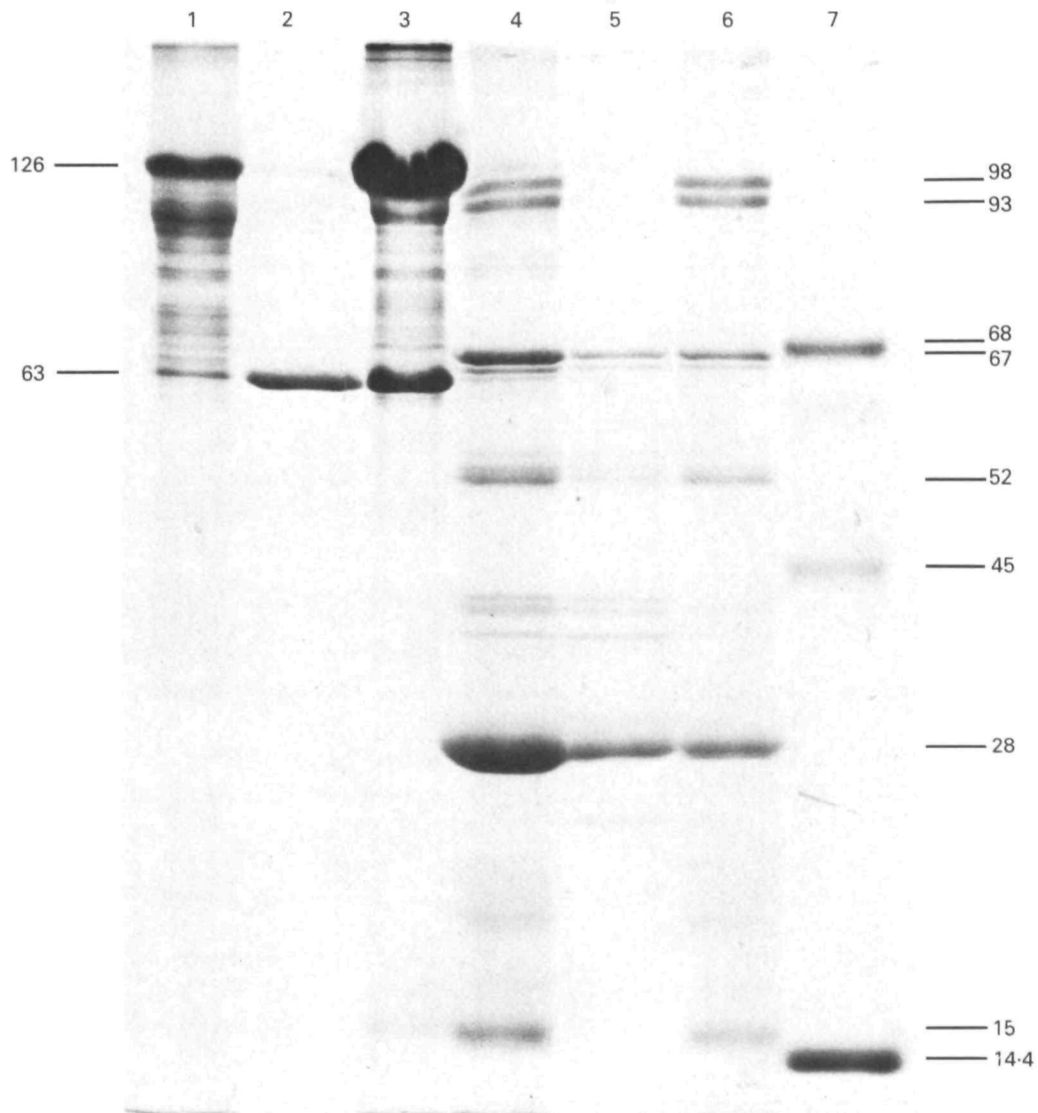


Fig. 1. SDS/13% polyacrylamide gel electrophoresis, Coomassie Blue-stained. Track 1, *B. thuringiensis var kurstaki* soluble crystal protein and track 2, *var kurstaki* insoluble crystal protein obtained by incubation of 100 µg of native crystal  $\delta$ -endotoxin in 50 mM- $\text{Na}_2\text{CO}_3 \cdot \text{HCl}$  (pH 9.5), 10 mM-dithiothreitol; track 3, 100 µg native *B. thuringiensis var kurstaki* crystal  $\delta$ -endotoxin; track 4, 100 µg native *B. thuringiensis var israelensis* crystal  $\delta$ -endotoxin; track 5, *var israelensis* soluble crystal protein and track 6, *var israelensis* insoluble crystal protein obtained by incubation of 100 µg of native crystal  $\delta$ -endotoxin in 50 mM- $\text{Na}_2\text{CO}_3 \cdot \text{HCl}$  (pH 10.5); track 7, molecular weight standards ( $\times 10^{-3}$ ): 50 µg bovine serum albumin (68 000), ovalbumin (45 000) and lysozyme (14 400).

Fig. 2. Phase contrast light micrographs of *A. albopictus* cells treated with 10 µl/ml 50 mM- $\text{Na}_2\text{CO}_3 \cdot \text{HCl}$  (pH 10.5) for 30 min (A); *A. albopictus* cells treated with 5 µg soluble *var israelensis* protein/ml for 20 min (B); *C. fumiferana* 63 CFI cells treated with 25 µl/ml 50 mM- $\text{Na}_2\text{CO}_3 \cdot \text{HCl}$  (pH 10.5) for 120 min (C); *C. fumiferana* 63 CFI cells treated with 25 µg soluble *var israelensis* protein/ml for 120 min (D). *B. thuringiensis var israelensis* soluble protein was obtained by incubation of native crystal  $\delta$ -endotoxin in 50 mM- $\text{Na}_2\text{CO}_3 \cdot \text{HCl}$  (pH 10.5). Bars, 25 µm.

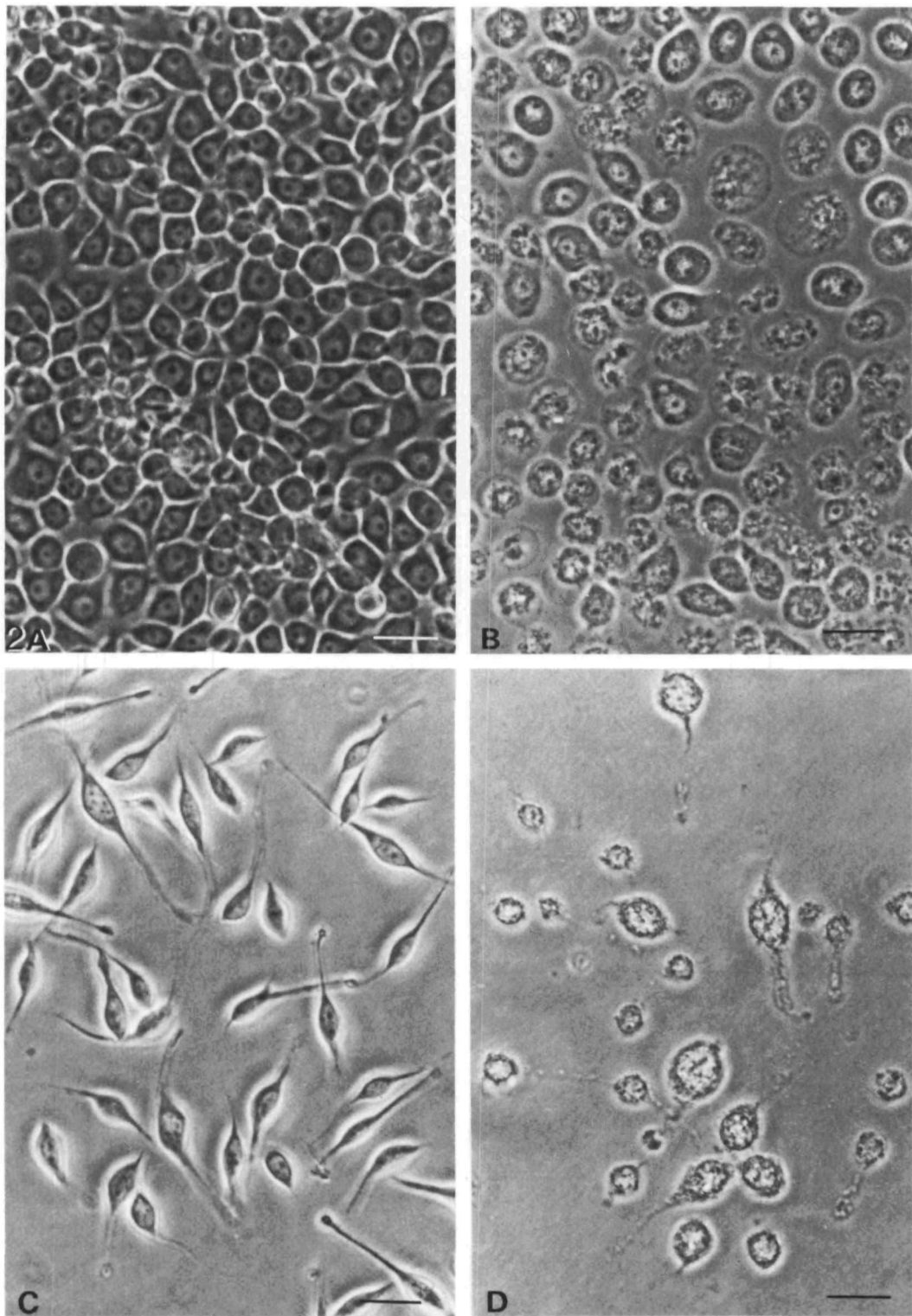


Fig. 2

the addition of 10 mM-dithiothreitol to the alkali-solubilization regime for *B. thuringiensis var israelensis* resulted in the solubilization of the 98 000 and 93 500  $M_r$  polypeptides (data not shown).

*Effect of soluble crystal  $\delta$ -endotoxin on insect and mammalian cells*

Soluble *B. thuringiensis var israelensis* crystal  $\delta$ -endotoxin protein, obtained by extraction with 50 mM- $\text{Na}_2\text{CO}_3 \cdot \text{HCl}$  (pH 10.5) as described above, caused rapid rounding up of *A. albopictus* cells followed sequentially by swelling (Fig. 2B), blebbing of membrane vesicles from the exterior of swollen cells and finally cell lysis. A protein concentration of 5  $\mu\text{g}/\text{ml}$  caused complete cell lysis in 30–40 min. Higher concentrations of the soluble *israelensis* crystal protein (25  $\mu\text{g}/\text{ml}$ ) were required to trigger the same cytological sequence (Fig. 2D) in *C. fumiferana* 63 CFI cells with essentially complete lysis occurring after 120 min. Using Trypan Blue staining as a measure of cell integrity the effect of these soluble proteins was demonstrable at earlier times. After 3 min and 10 min, 66% and 93%, respectively, of *A. albopictus* cells ( $2 \times 10^6/\text{ml}$ ) were found to be non-viable after exposure to 5  $\mu\text{g}$  soluble *israelensis* protein/ml (Fig. 3). *C. fumiferana* 63 CFI cells ( $10^6/\text{ml}$ ) exposed to 25  $\mu\text{g}$  soluble protein/ml were 25% and 65% non-viable after 15 min and 47 min, respectively (Fig. 4).

The *israelensis* crystal  $\delta$ -endotoxin protein soluble in 50 mM- $\text{Na}_2\text{CO}_3 \cdot \text{HCl}$  (pH 10.5) triggered a similar sequence of cytological changes in two other insect cell lines, *S. frugiperda* and *T. ni* (Fig. 5B,D).

Studies with a variety of mammalian cell types revealed that mouse fibroblasts, primary pig lymphocytes and three mouse epithelial carcinoma cell lines (Fig. 5F)

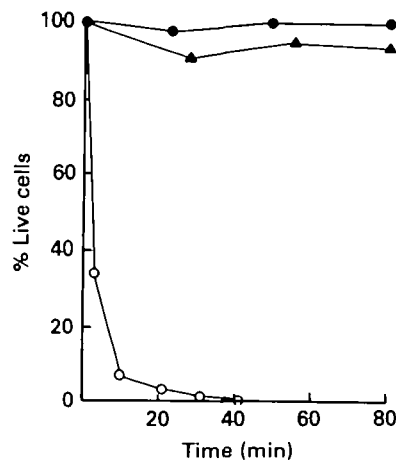


Fig. 3. Percentage viable *A. albopictus* cells ( $2 \times 10^6/\text{ml}$ ) remaining, as measured by Trypan Blue staining, after treatment with: 5  $\mu\text{l}/\text{ml}$  of 50 mM- $\text{Na}_2\text{CO}_3 \cdot \text{HCl}$  (pH 10.5) (●—●); 10  $\mu\text{g}$  protein/ml of native *B. thuringiensis var israelensis* crystal  $\delta$ -endotoxin (▲—▲); and 5  $\mu\text{g}$  protein/ml of *var israelensis* crystal  $\delta$ -endotoxin protein soluble in 50 mM- $\text{Na}_2\text{CO}_3 \cdot \text{HCl}$  (pH 10.5) (○—○).



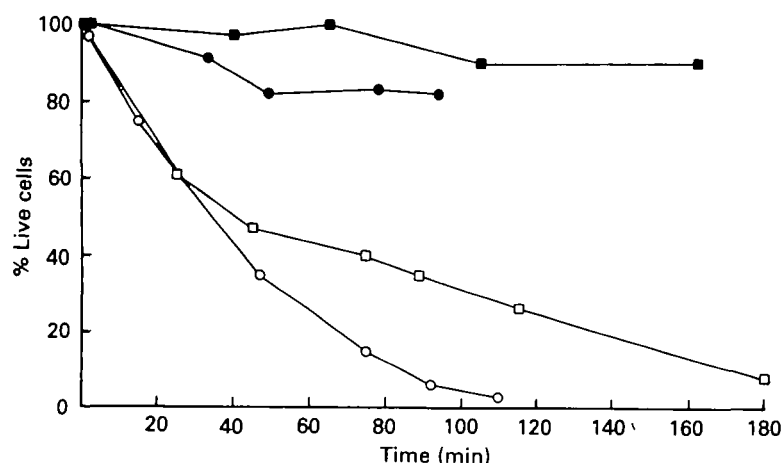


Fig. 4. Percentage viable *C. fumiferana* cells remaining as measured by Trypan Blue staining.  $10^6$  cells/ml treated with  $25 \mu\text{l/ml}$  of  $50 \text{ mM-Na}_2\text{CO}_3 \cdot \text{HCl}$  (pH 10.5) (●—●), and  $25 \mu\text{g protein/ml}$  of *B. thuringiensis var israelensis* crystal  $\delta$ -endotoxin protein soluble in  $50 \text{ mM-Na}_2\text{CO}_3 \cdot \text{HCl}$  (pH 10.5) (○—○);  $2 \times 10^6$  cells/ml treated with  $25 \mu\text{l/ml}$  of  $50 \text{ mM-Na}_2\text{CO}_3 \cdot \text{HCl}$  (pH 9.5) (■—■), and  $25 \mu\text{g protein/ml}$  *B. thuringiensis var kurstaki* crystal  $\delta$ -endotoxin protein soluble in  $50 \text{ mM-Na}_2\text{CO}_3 \cdot \text{HCl}$  (pH 9.5),  $10 \text{ mM-dithiothreitol}$ , precipitated with saturated ammonium sulphate, and resuspended ( $2 \mu\text{g}/\mu\text{l}$ ) in  $50 \text{ mM-Na}_2\text{CO}_3 \cdot \text{HCl}$  (pH 9.5) (□—□).

were also susceptible to similar concentrations of the soluble *israelensis* crystal  $\delta$ -endotoxin protein. In contrast to these results, protoplasts prepared from the gram-positive bacterium *B. megaterium* KM, when viewed microscopically, appeared to be unaffected by high concentrations ( $0.4 \text{ mg/ml}$ ) of the crystal  $\delta$ -endotoxin protein soluble in  $50 \text{ mM-Na}_2\text{CO}_3 \cdot \text{HCl}$  (pH 10.5).

No *in vitro* effects were observed when the various insect and mammalian cell types, or *B. megaterium* KM protoplasts, were exposed to either the same concentration of native crystal  $\delta$ -endotoxin or the same volume of  $50 \text{ mM-Na}_2\text{CO}_3 \cdot \text{HCl}$  (pH 10.5) buffer (Fig. 2A,C; Fig. 5A,C,E).

It was found that the soluble *B. thuringiensis var kurstaki* crystal  $\delta$ -endotoxin protein prepared by a similar extraction procedure (Huber *et al.* 1981) was toxic only to *C. fumiferana* 63 CFI cells. All other cell types so far described appeared unaffected. The *C. fumiferana* 63 CFI cells exhibited the typical sequence of cytological changes; swelling, blebbing and eventual cell lysis as previously reported by Murphy *et al.* (1976), Nishiitsutsuji-Uwo *et al.* (1979) and Geiser (1979). In a typical experiment with *C. fumiferana* 63 CFI cells,  $55 \mu\text{g/ml}$  of soluble protein caused total cell lysis of  $2 \times 10^6$  cells/ml in 200 min (Fig. 4).

#### Haemolytic activity

The soluble crystal  $\delta$ -endotoxin protein from *B. thuringiensis var israelensis* prepared by incubation with  $50 \text{ mM-Na}_2\text{CO}_3 \cdot \text{HCl}$  (pH 10.5) was found to be haemolytic for horse, human, sheep, rat, mouse and rabbit erythrocytes, at room temperature. No haemolytic activity could be detected when the protein was incubated

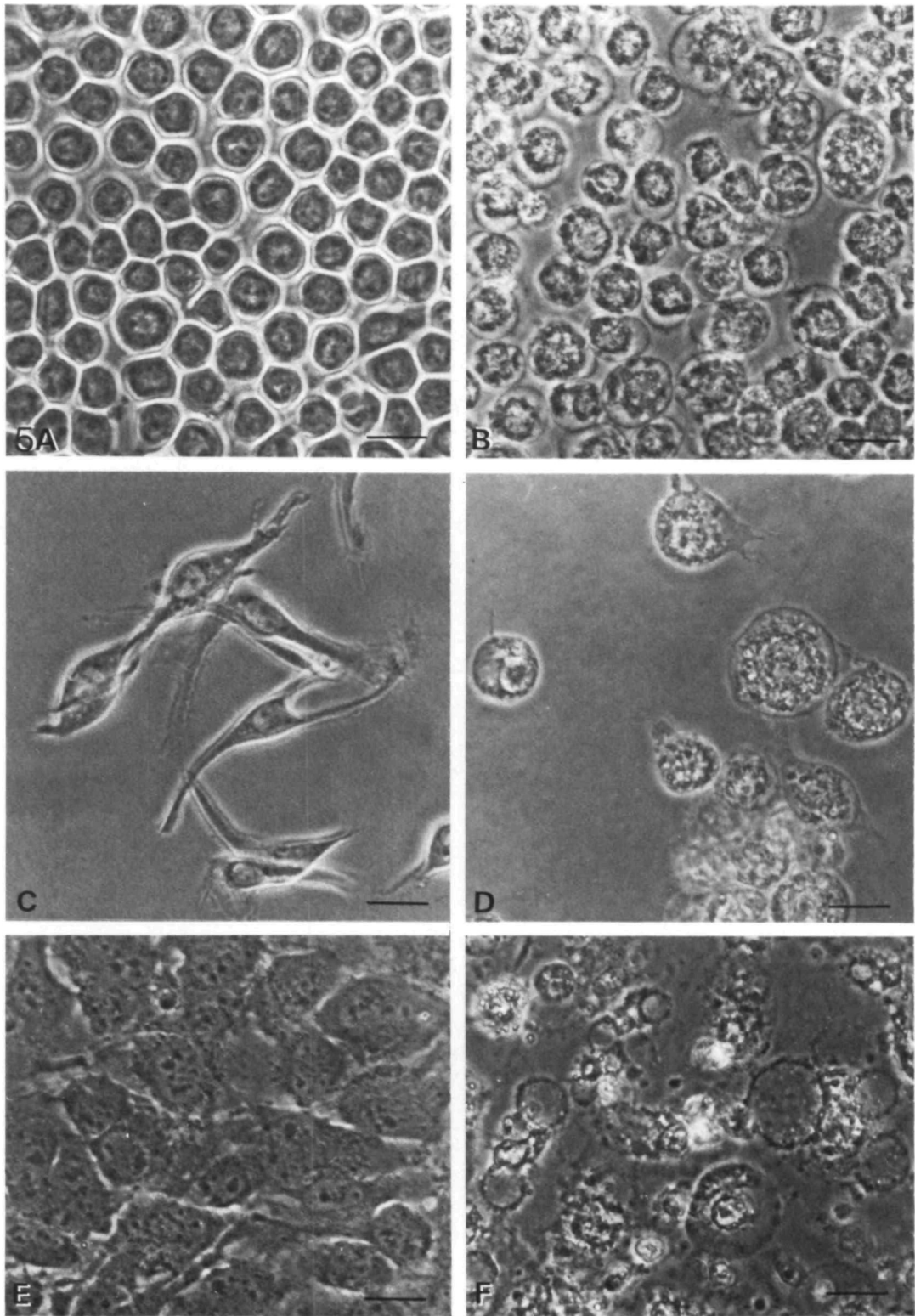


Fig. 5

with erythrocytes at 4°C. However, when the microtitre plates, which had been at 4°C, were subsequently incubated at room temperature, haemolysis was observed within 3 h. When the haemolytic activity of the crystal  $\delta$ -endotoxin protein soluble in 50 mM- $\text{Na}_2\text{CO}_3\cdot\text{HCl}$  (pH 10.5) was titrated by twofold serial dilution, using a standardized suspension of horse, human and sheep erythrocytes as previously described, complete haemolysis occurred down to dilutions of 1:16, 1:64 and 1:2, respectively. No haemolysis was observed at room temperature when the erythrocytes were challenged with any of the other test samples (see Materials and Methods). None of the test samples of *B. thuringiensis var kurstaki* caused haemolysis of erythrocytes.

#### Effect of soluble crystal $\delta$ -endotoxin in vivo

Bioassay of native *B. thuringiensis var israelensis* crystal  $\delta$ -endotoxin, and the soluble and insoluble material obtained after incubation of the crystal  $\delta$ -endotoxin in 50 mM- $\text{Na}_2\text{CO}_3\cdot\text{HCl}$  (pH 10.5) in *A. aegypti* 3rd instar larvae (Tyrell *et al.* 1979) resulted in an  $\text{LC}_{50}$  of 5–50 ng protein/ml, 0.6–6  $\mu\text{g}$  protein/ml and 50–500 ng protein/ml, respectively. *P. brassicae* 4th instar larvae fed these samples per os (2  $\mu\text{g}$  protein/larvae) showed no inhibition of feeding and no other apparent ill effects (Table 1).

The results of experiments with Balb.c mice are summarized in Table 1. In a series of preliminary toxicity tests with adult and suckling Balb.c mice the native *B. thuringiensis var israelensis* and *var kurstaki* crystal  $\delta$ -endotoxins produced no pathological effects when introduced by intravenous inoculation, or when fed per os. Although the *israelensis* crystal  $\delta$ -endotoxin protein soluble in 50 mM- $\text{Na}_2\text{CO}_3\cdot\text{HCl}$  (pH 10.5) had no effect when fed per os to adult mice (0.5 mg protein/animal), or suckling mice (200  $\mu\text{g}$  protein/animal) it was found to be toxic when introduced by intravenous inoculation. Adult Balb.c mice (average body weight 30 g) were killed within 12 h by intravenous inoculation of 1.0 mg of *israelensis* soluble crystal  $\delta$ -endotoxin protein. Within 1 h of injection animals developed paralysis in their hind-quarters and became relatively immobile within 3 h. Their breathing and heart-rate also increased. Animals inoculated intravenously with 0.5 mg of soluble protein developed similar paralysis in their hind-quarters, but those animals that died, did so after 36–48 h. Although none of the animals inoculated intravenously with 0.1 mg of soluble protein died, three animals developed paralysis in their left hind-leg and a further three showed a stiffening of movement in their hind-quarters. Suckling mice (average body weight 5.7 g) were killed within 3 h by subcutaneous inoculations of 80–200  $\mu\text{g}$  protein/animal. A

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Fig. 5. Phase-contrast light micrographs of *S. frugiperda* cells treated with 20  $\mu\text{l}$ /ml 50 mM- $\text{Na}_2\text{CO}_3\cdot\text{HCl}$  (pH 10.5) for 40 min (A), and treated with 20  $\mu\text{g}$  protein/ml of soluble *var israelensis* crystal  $\delta$ -endotoxin for 40 min (B); *T. ni* cells treated with 10  $\mu\text{l}$ /ml of 50 mM- $\text{Na}_2\text{CO}_3\cdot\text{HCl}$  (pH 10.5) for 15 min (C), and treated with 10  $\mu\text{g}$  protein/ml of soluble *var israelensis* crystal  $\delta$ -endotoxin for 15 min (D); mouse epithelial carcinoma cells (EC 6) treated with 25  $\mu\text{l}$ /ml 50 mM- $\text{Na}_2\text{CO}_3\cdot\text{HCl}$  (pH 10.5) for 30 min (E), and treated with 25  $\mu\text{g}$  protein/ml of soluble *var israelensis* crystal  $\delta$ -endotoxin for 30 min (F). *B. thuringiensis var israelensis* soluble protein was obtained by incubation of native crystal  $\delta$ -endotoxin in 50 mM- $\text{Na}_2\text{CO}_3\cdot\text{HCl}$  (pH 10.5). Bars, 25  $\mu\text{m}$ .

Table 1. In vivo assays

Test sample	Amount of protein per animal	Test animal	Method of administration	No. dead/Total no.
Native <i>B. thuringiensis</i> var <i>israelensis</i> crystal $\delta$ -endotoxin in PBS	1.0 mg	Adult mice	i.v.	0/5
	0.5 mg	Adult mice	i.v.	0/5
	0.5 mg	Adult mice	per os	0/3
	0.1 mg	Adult mice	i.v.	0/10
	80–200 $\mu$ g	Suckling mice	s.c.	0/15
	200 $\mu$ g	Suckling mice	per os	0/3
	2 $\mu$ g	<i>P. brassicae</i> larvae	per os	0/10
Native <i>B. thuringiensis</i> var <i>kurstaki</i> crystal $\delta$ -endotoxin in PBS	1.0 mg	Adult mice	i.v.	0/5
	0.5 mg	Adult mice	i.v.	0/5
	0.5 mg	Adult mice	per os	0/4
	80–200 $\mu$ g	Suckling mice	s.c.	0/13
	2 $\mu$ g	<i>P. brassicae</i> larvae	per os	10/10
<i>B. thuringiensis</i> var <i>israelensis</i> soluble* crystal protein in 50 mM-Na <sub>2</sub> CO <sub>3</sub> ·HCl (pH 10.5)	1.0 mg	Adult mice	i.v.	7/7
	0.5 mg	Adult mice	i.v.	3/5
	0.5 mg	Adult mice	per os	0/3
	0.1 mg	Adult mice	i.v.	0/10†
	80–200 $\mu$ g	Suckling mice	s.c.	16/16
	200 $\mu$ g	Suckling mice	per os	0/8
	2 $\mu$ g	<i>P. brassicae</i> larvae	per os	0/10
<i>B. thuringiensis</i> var <i>kurstaki</i> soluble‡ crystal protein in 50 mM-Na <sub>2</sub> CO <sub>3</sub> ·HCl (pH 9.5)	1.0 mg	Adult mice	i.v.	0/10
	0.5 mg	Adult mice	i.v.	0/5
	0.5 mg	Adult mice	per os	0/5
	80–200 $\mu$ g	Suckling mice	s.c.	0/3
	200 $\mu$ g	Suckling mice	per os	0/5
	2 $\mu$ g	<i>P. brassicae</i> larvae	per os	10/10
0.4 ml 50 mM-Na <sub>2</sub> CO <sub>3</sub> ·HCl (pH 10.5)	—	Adult mice	i.v.	0/15
50 $\mu$ l 50 mM-Na <sub>2</sub> CO <sub>3</sub> ·HCl (pH 10.5)	—	Suckling mice	per os	0/5
0.4 ml 50 mM-Na <sub>2</sub> CO <sub>3</sub> ·HCl (pH 9.5)	—	Adult mice	i.v.	0/7
0.2 ml 50 mM-Na <sub>2</sub> CO <sub>3</sub> ·HCl (pH 9.5)	—	Suckling mice	s.c.	0/13

\* Crystal  $\delta$ -endotoxin protein solubilized in 50 mM-Na<sub>2</sub>CO<sub>3</sub>·HCl (pH 10.5).

† Six of the mice showed paralysis, or stiffness in their hind-quarters.

‡ Crystal  $\delta$ -endotoxin protein solubilized in 50 mM-Na<sub>2</sub>CO<sub>3</sub>·HCl (pH 9.5), 10 mM-dithiothreitol. i.v., intravenous inoculation into the tail vein; s.c., subcutaneous inoculation into left thigh. White Balb.c mice were used. *P. brassicae* 4th instar larvae were used.

severe haematoma was observed in the area around the site of injection. This effect was not observed in animals receiving control injections.

In contrast to these results, native *B. thuringiensis* var *kurstaki* crystal  $\delta$ -endotoxin and soluble protein prepared by incubation of *B. thuringiensis* var *kurstaki* crystal  $\delta$ -endotoxin in 50 mM-Na<sub>2</sub>CO<sub>3</sub>·HCl (pH 9.5), 10 mM-dithiothreitol produced no pathology in adult Balb.c mice (1.0 mg/animal), or suckling Balb.c mice (200  $\mu$ g/animal) by intravenous, or subcutaneous inoculation, or by feeding per os.

## DISCUSSION

The molecular basis of the insecticidal activity of the crystal  $\delta$ -endotoxin produced by *B. thuringiensis* serotypes is not known. Previous studies of *B. thuringiensis* serotypes active against Lepidoptera have demonstrated a characteristic disruptive effect on insect gut epithelium *in vivo* (Angus, 1970; Ebersold *et al.* 1980; Endo & Nishiitsutsuji-Uwo, 1980). The native crystal  $\delta$ -endotoxin is non-toxic until ingested by susceptible insects. Pathogenicity appears to involve dissociation of the crystal  $\delta$ -endotoxin in the alkaline conditions of the insect gut and/or proteolytic modification by gut enzymes to yield active toxin (Faust, Hallam & Travers, 1974; Lecadet, 1970). Activation of the crystal  $\delta$ -endotoxin has been achieved *in vitro* by incubation of the crystals with gut proteases (Lecadet & Dedonder, 1966; Lecadet & Martouret, 1967). Crystal  $\delta$ -endotoxin from *B. thuringiensis var kurstaki* and *var aizawai*, activated in this way, produced characteristic cytopathological effects on certain insect cell lines *in vitro* (Murphy *et al.* 1976; Nishiitsutsuji-Uwo *et al.* 1979; Johnson, 1981). These and other data suggest that the plasma membrane of susceptible cells may be the primary target for the  $\delta$ -endotoxin (Fast, Sohi & Murphy, 1978). Subsequent cell lysis may be the result of specific inhibition of certain membrane proteins responsible for the maintenance of the correct  $K^+$  balance across the midgut membrane (Griego, Moffet & Spence, 1979; Harvey & Wolfersberger, 1979; Cioffi & Harvey, 1981). Alternatively, the crystal  $\delta$ -endotoxin may resemble the cytolytic bacterial toxins such as *Staphylococcus aureus*  $\delta$ -toxin in its mode of action (Thelestam & Moellby, 1975*a,b*).

The recently isolated *israelensis* strain of *B. thuringiensis* has excited considerable interest because of its pathogenicity for the larvae of certain Diptera including the genera *Aedes* and *Culex* (Goldberg & Margalitt, 1977), which are vectors of malaria and filariasis, respectively. In a study of the *in vivo* effects of the *israelensis* crystal  $\delta$ -endotoxin on mosquito larvae, de Barjac (1978*b*) demonstrated the typical swelling and lysis of gut cells that characterises the action of crystal  $\delta$ -endotoxin from other *B. thuringiensis* serotypes on Lepidopteran gut cells. To our knowledge the present report is however, the first description of an investigation of the *in vitro* cytopathological and haemolytic effects of the *israelensis* crystal  $\delta$ -endotoxin.

It has been reported that the varieties of *B. thuringiensis* crystal  $\delta$ -endotoxin can be solubilized by alkaline buffer (pH > 12) (Bulla *et al.* 1981), or a combination of alkaline buffer (pH 9–10) and reducing conditions (Huber *et al.* 1981). Crystal  $\delta$ -endotoxin preparations solubilized by these methods retained *in vivo* toxicity, but to date *in vitro* toxicity of these soluble preparations on isolated cell cultures has not been observed unless the alkali-treated toxin is additionally exposed to proteolytic enzymes (Fast, 1981). A similar alkaline regime was developed and used in the present study to solubilize *B. thuringiensis var israelensis* crystal  $\delta$ -endotoxin. By incubating *israelensis* crystal  $\delta$ -endotoxin with 50 mM- $Na_2CO_3 \cdot HCl$  (pH 10.5) for 30 min at 37 °C, 40% of the crystal protein was extracted in a soluble form, which retained toxicity *in vivo* and *in vitro*. Analysis of the solubilized polypeptides by SDS/polyacrylamide gel electrophoresis (Fig. 1, track 5) revealed that a substantial amount of the major

*israelensis* crystal  $\delta$ -endotoxin polypeptide ( $M_r$  28 000) was extracted by this method. By contrast, the two high molecular weight polypeptides ( $M_r$  98 000 and 93 500) remained completely insoluble. It is interesting to note certain similarities between the crystal  $\delta$ -endotoxin and the granulosis and nuclear polyhedrosis insect viruses. In both cases the primary site of action is the insect gut epithelium. Summers & Egawa (1973) reported the solubilization of a protein ( $M_r$  180 000) from the crystalline proteinaceous structure that surrounds the granulosis virus of *T. ni* using a 70 mM-sodium carbonate buffer (pH 10.7). Also, Eppstein, Thoma, Scott & Young (1975) reported that the crystalline matrix protein ( $M_r$  28 000) of the nuclear polyhedrosis virus of *T. ni* could be solubilized by incubation with 100 mM-sodium carbonate buffer (pH 11). For these occluded viruses, therefore, the alkaline insect gut environment is also thought to be a factor in the solubilization of viral proteins, and the subsequent release of infective particles into the gut during the primary infection (Harrap, 1970). However, these proteins do not appear to have the characteristic effect on gut cells seen with *B. thuringiensis*.

The *israelensis* crystal  $\delta$ -endotoxin soluble in 50 mM- $\text{Na}_2\text{CO}_3 \cdot \text{HCl}$  (pH 10.5) retained toxicity for mosquito larvae and produced rapid cytopathological changes in all the insect cell lines examined. There was clearly a difference between the  $\text{LC}_{50}$  for mosquitos of intact *israelensis* crystal  $\delta$ -endotoxin and the soluble crystal protein. This may reflect the fact that the larvae are filter feeders and that the particulate crystals are more efficiently ingested than the alkali-solubilized toxin. Alternatively, the *in vitro* alkali-mediated solubilization/activation regime used in this study may only partially simulate the *in vivo* activation, which requires a combination of the high pH of the insect gut and proteolytic cleavage by gut proteases.

The observed cytopathology paralleled that observed by several authors using protease-activated crystal  $\delta$ -endotoxin from other *B. thuringiensis* serotypes (Murphy *et al.* 1976; Geiser, 1979; Nishiitsutsuji-Uwo, Endo & Himeno, 1980). The latter authors reported no cytopathic effect of the activated  $\delta$ -endotoxin on mammalian cells. However, Prasad & Shetna, (1976) and Seki *et al.* (1978) have reported cytopathological effects of *B. thuringiensis var thuringiensis* crystal  $\delta$ -endotoxin on the Yoshida ascites sarcoma tumour line maintained in Wistar rats and sarcoma 180 ascites cells. It is significant, therefore, that in the present study the alkali-soluble *israelensis*  $\delta$ -endotoxin was lethal to the various mammalian cell lines examined. In addition this  $\delta$ -endotoxin showed haemolytic activity against a wide range of erythrocytes. Interestingly, only one cell type, viz. bacterial protoplasts (derived from an organism unrelated to the producer organism), failed to show the typical cytopathic response to the soluble *israelensis* crystal  $\delta$ -endotoxin.

In parallel studies with the crystal  $\delta$ -endotoxin from *B. thuringiensis var kurstaki* it was found that a reducing agent was additionally required to achieve solubilization (90%) in 50 mM- $\text{Na}_2\text{CO}_3 \cdot \text{HCl}$  (pH 9.5). In this respect there are again similarities in the conditions required for solubilization of the crystalline bacterial toxin and the inclusion-body protein of the Entomopox insect viruses, which also requires a reducing agent in addition to dilute alkali for dissolution (Vaughn, 1974). The solubilized preparation retained its toxicity to Lepidoptera *in vivo* and produced the typical

cytological effect on *C. fumiferana* 63 CFl cells *in vitro*. However, it was without effect on mosquito-derived cells, mammalian cells, bacterial protoplasts, or the other Lepidopteran cell lines. Moreover, it showed no haemolytic activity against the range of erythrocytes. It would appear that the *in vitro* activity of the solubilized *kurstaki* crystal  $\delta$ -endotoxin is restricted in its host range to cells derived from susceptible insects. In contrast, the data for the *israelensis* crystal  $\delta$ -endotoxin indicate that provided it is rendered soluble by incubation with 50 mM- $\text{Na}_2\text{CO}_3 \cdot \text{HCl}$  (pH 10.5) it is toxic not only to mosquito cells, but also to a wide variety of cell types outside its *in vivo* host range, including mammalian cells and erythrocytes.

The failure of the *israelensis* toxin to affect bacterial protoplasts, or to kill 4th instar *P. brassicae* larvae, suggests that the bacterial plasma membrane and the *Lepidopteran* gut epithelium may lack the target/receptor for the soluble  $\delta$ -endotoxin. Alternatively, the resistance of the Lepidoptera may reflect some inhibitory effect of the larval gut contents on the toxin.

The above differences in the spectrum of activity of *israelensis* and *kurstaki* alkali-solubilized crystal  $\delta$ -endotoxins were reinforced by the results of mammalian *in vivo* toxicity tests. The native crystal  $\delta$ -endotoxin from either *israelensis* or *kurstaki* produced no ill effects in Balb.c mice when administered per os, subcutaneously, or intravenously. Similarly the solubilized *kurstaki* crystal  $\delta$ -endotoxin was also non-toxic when administered by these routes. Although the soluble *israelensis* crystal  $\delta$ -endotoxin was without effect when administered per os, it proved to be extremely toxic when inoculated intravenously into Balb.c mice, or subcutaneously into suckling Balb.c mice, at doses of 15–35  $\mu\text{g}$  soluble protein/g body weight.

Although the 28 000  $M_r$  polypeptide is the major component of the *israelensis*  $\delta$ -endotoxin protein soluble in 50 mM- $\text{Na}_2\text{CO}_3 \cdot \text{HCl}$  (pH 10.5), we cannot at present correlate the observed insect and mammalian cell toxicity and haemolytic activity solely with this component. It is possible that the different lytic activities may reside on different polypeptides. Further purification of this soluble preparation is being carried out to resolve this question.

In view of the obvious potential of *B. thuringiensis var israelensis* as a biological control agent it is important to emphasize that although toxic effects resulted from inoculation of the alkali-soluble *israelensis*  $\delta$ -endotoxin into mice, the same preparations produced no observable ill effects when administered orally. This important observation is verified by the absence of *in vivo* toxicity of this variety in oral tests with a large number of non-target animals, in which only groups very closely related to mosquitos (e.g. chironomids) were affected (Burgess, 1982). The native crystal  $\delta$ -endotoxin and spores have also been injected parenterally into many test animal species without effect. It would be of value to examine the effect of parenteral application of the soluble toxin of more strains on a range of animal species.

The broad spectrum of toxicity shown by the *israelensis* toxin clearly distinguishes it from the *kurstaki* toxin and suggests that the molecular mechanisms of the two toxins in Lepidoptera and Diptera may be quite different. It will now be important to determine to what extent the other *B. thuringiensis* strains, reported to be pathogenic for Diptera, display the same spectrum of toxicity observed here for *var israelensis*.

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