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The Apparent *in Vivo* Neuromuscular Effects of the δ-Endotoxin of *Bacillus thuringiensis* var. *israelensis* in Mice and Insects of Four Orders

PETER Y. K. CHEUNG,[1] RICHARD M. ROE, BRUCE D. HAMMOCK, CHARLES L. JUDSON, AND MARY ANN MONTAGUE

Departments of Entomology and Environmental Toxicology, University of California, Davis, California 95616

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Alkaline-dissolved crystal δ-endotoxin from *Bacillus thuringiensis* var. *israelensis* (serovar H 14) was injected into mice and seven species of insects representing the orders Lepidoptera, Orthoptera, Coleoptera, Hemiptera, and Diptera. High *in vivo* toxicity, at 1 to 5 ppm (μg toxin/g body wet wt), was observed with mice and some insects, including some that are not sensitive to the toxin when administered orally. Neuromuscular effects were observed when the toxin was injected directly into the body cavity of the test animals. Biochemical studies suggested that different protein fragments within the crystal δ-endotoxin may be responsible for the majority of the mosquito larvacidal activity and the neurotoxic symptoms observed in larvae of *Trichoplusia ni*.

INTRODUCTION

The gram-positive bacterium *Bacillus thuringiensis* (BT) is characterized by the larvacidal properties of its proteinaceous parasporal crystal, formed during the sporulation of the microorganism (1). The crystal toxins (or the δ-endotoxins) from most of the *B. thuringiensis* serotypes are toxic to larvae of Lepidoptera upon ingestion. These include the subspecies *kurstaki* (BT *kurstaki*), *thuringiensis*, *tolworthi*, and many others. On the other hand, the one strain, *B. thuringiensis* var. *israelensis* [designated serovar H14 (2, 3)], differs from the others by being highly toxic toward certain dipteran larvae, particularly mosquitoes and black flies (3, 4), but not against Lepidoptera.

Reports up to now have shown that the crystal toxin, upon ingestion, is quickly activated by a combination of the alkaline gut pH and proteolytic enzymes present in the mid-gut of the insect (5–7). Subsequent histopathological observations include swelling and vacuolization of epithelial cells, separation of these cells from the basement membrane and from each other, and eventually disruption of the gut–hemocoel barrier (8–11). With *B. thuringiensis* var. *israelensis* (BT *israelensis*), similar histopathological changes were reported when mosquito larvae were exposed to the δ-endotoxin of the microorganism (12). In addition, the caeca of *Aedes aegypti* (L.) (Diptera: Culicidae) and *Simulium vittatum* (Zetterstedt) (Diptera: Simuliidae) were reported to be affected in the same way (13, 14).

These accumulated observations have led to the general acceptance that the action of the δ-endotoxin is directed against, if not restricted to, the gut epithelium of the host insect (8, 9). However, the reported cytotoxicity of the BT *israelensis* δ-endotoxin on cultured insect cells should have suggested a broader spectrum of target tissues for the toxin (15–17). The most recent report by Thomas and Ellar (18) has shown that alkaline-dissolved δ-endotoxin from BT *israelensis* is cytotoxic to four insect and five mammalian cultured cell lines. The soluble toxin is also hemolytic toward var-

[1] To whom inquiries should be addressed.

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ious mammalian erythrocytes and is fairly toxic toward mice when injected directly. Further studies by the same authors showed that the toxin has a strong affinity for specific phospolipids of the plasma membrane, thus suggesting an insecticidal mechanism for the BT *israelensis* toxin (19).

In this paper bioassays based on comparative toxicity of BT *israelensis* to larvae of *A. aegypti* and *Trichoplusia ni* (Hubner) (Lepidoptera: Noctuidae) were used to monitor the purification of the polypeptides apparently responsible for the toxic effects of BT *israelensis*. The *in vivo* toxicity of carbonate-dissolved BT *israelensis* and BT *kurstaki* δ-endotoxin on several species of insects were compared to demonstrate the differences of the two toxins.

MATERIALS AND METHODS

Microorganisms and growth conditions. *B. thuringiensis* var. *israelensis* was isolated from a commercial preparation provided by Sandoz Inc. The organism was maintained on a GYS[2] (20) agar slant at 4°C and subcultured every 3 months. Cells for experimental use were cultured as follows. GYS medium (100 ml) was inoculated with the organism and incubated overnight on a rotary shaker (200 rpm). When the purity of the culture was confirmed by phase-contrast microscopic examination (BT *israelensis* and BT *kurstaki* crystals differ in morphology), it was transfered into a 2-liter Erlenmeyer flask containing 1 liter of modified GYS medium. The flask was incubated at 30°C and aerated by rotary agitation at 200 rpm for 72 to 84 hr. At the end of the incubation period, spores and parasporal crystals were released by autolysis. Spores and crystals were collected by centrifugation at 5000 rpm for 20 min (Sorvall, GSA rotor). BT *israelensis* strain IFC-1 and BT *kurstaki* were provided by Biochem Products-US Division (Salsbury Labs., Inc.). Cell debris and putative surface-bound protease were removed by washing with 1 *M* NaCl (2×) and distilled water (3×), and the spore/crystal mixture was stored at −20°C.

Purification of crystal δ-endotoxin. BT *israelensis* crystal toxin was separated from the spores by Renografin density gradient centrifugation (2×) as described by Sharpe *et al.* (21). BT *kurstaki* crystal toxin was purified by discontinuous sucrose density gradient centrifugation (2×) as described by Thomas and Ellar (18). Purity of the crystal preparations was monitored by examination under the phase-contrast microscope.

Protein determination. Protein concentrations of samples were determined by the method of Lowry *et al.* (22). Other protein assaying methods, Bio Rad and Biuret, were inconsistent when employed in assaying the protein concentrations of alkaline-dissolved δ-endotoxin.

Dissolution of crystal δ-endotoxin. Parasporal crystals from density gradient centrifugation were washed with distilled water 3× to remove the Renografin or sucrose. The pellet was then suspended in 0.5% sodium carbonate (pH 11.0) and 0.02% sodium azide for 3 hr at 28°C with sonication at 30-min intervals. Purity of the solubilized toxin was monitored by using enzyme-linked immunosorbent assays (ELISAs) developed for the BT *israelensis* and BT *kurstaki* crystal proteins (23). The carbonate-dissolved toxin was then dialyzed overnight against 0.025 *M* phosphate buffer, pH 8.0, sterile-filtered, and divided into aliquots before being frozen at −60°C.

Polyacrylamide gel electrophoresis. Discontinuous SDS–polyacrylamide gel electrophoresis (SDS-PAGE), 12.5%, was carried out as described by Laemmli (24) with a Hoefer vertical slab gel system (SE 600, Hoefer Scientific Instruments). Molecular markers included bovine serum albumin (68,000), egg albumin (43,000), trypsin

[2] Abbreviations used: GYS, glucose/yeast extract/sodium citrate; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; L:D, light:dark; PI, postinoculation; PBS, physiological buffered saline; ppm, μg toxin/g body wet weight; V_e/V_o, elution volume/void volume.

(24,000), and myoglobin (17,000). Gels were stained with 0.1% Coomassie brilliant blue in ethanol:water:acetic acid (33:11:3.6) and destained in the same solvent.

DEAE anion-exchange chromatography. Dissolved BT *israelensis* toxin was applied onto a DEAE-Cellulose (DE-52, Whatman) column (30 ml bed volume), preequilibrated at pH 8.00 with a 0.025 *M* Tris–HCl buffer. After washing with 3 bed volumes of the starting buffer, the column was eluted with a sodium chloride gradient of 0.0 to 0.5 *M*.

Acid precipitation. The effluent from DEAE chromatography was dialyzed against a 0.05 *M* sodium acetate buffer, pH 4.5. The precipitates, when developed, were removed by centrifugation at 5000 rpm for 15 min (Sorvall, SS-34 rotor). The supernatant was dialyzed against 0.05 *M* phosphate buffer, pH 8.0.

Gel-filtration liquid chromatography. Sephadex G-75 superfine (100–200 mesh, Pharmacia) was swollen in 0.025 *M* sodium phosphate buffer, pH 8.0. A glass column of 95 × 1.3 cm was packed with the slurry. The Sephadex bed was further washed with 2 bed volumes of phosphate buffer. The void and inclusion volumes were determined by passing Blue Dextran (1×10^6) and *p*-nitrophenol through the column. Molecular markers included bovine serum albumin, ovalbumin, and myoglobin.

Test animals. Bioassays were conducted on a number of insect species and mice. These animals and their rearing conditions are described below.

Larvae of the yellow fever mosquito, *A. aegypti,* were reared at 28°C with a 16L:8D, photoperiod on a diet consisting of guinea pig chow (Geisler):liver powder (Sigma):Tetra Min (50:50:1, w/w). Third-stadium larvae were used for assays. Adult mosquitoes from the same colony were maintained at 28°C and fed 5% sucrose solution. Three to four days after emergence, female mosquitoes were used for experiments.

Larvae of the cabbage looper, *T. ni,* were reared on a diet modified from Shorey and Hale (25) by Roe *et al.* (26) at 27°C with a 14L:10D photoperiod. Two-day-old fifth-stadium larvae, having an average weight of 0.25 ± 0.02 g, were selected for experiments.

The corn earworm larvae, *Heliothis zea* (Boddie) (Lepidoptera: Noctuidae), were reared on Southwestern Corn Borer diet (Bioserv Inc., Frenchtown, N.J.; product No. 976L3) at room temperature with a 16L:8D photoperiod. Experimental larvae were 1 day into the fourth stadium, with an average weight of 0.35 ± 0.18 g.

Milkweek bugs, *Oncopeltus fasciatus* (Dallas) (Hemiptera: Lygaeidae), were maintained at 28°C on a diet consisting of sunflower seed and water with a 16L:8D photoperiod. Adults with an average weight of 0.040 ± 0.007 g were selected for injections.

Larvae of yellow mealworm, *Tenebrio molitor* (L.) (Coleoptera: Tenebrionidae), were reared at 28°C on a diet consisting of oatmeal and apple with a 16L:8D photoperiod. Larvae weighing 0.11 ± 0.03 g were used for injections.

Newly emerged house flies, *Musca domestica* (L.) (Diptera: Muscidae), were provided by Don deVris from Dow Chemical Company (Walnut Creek, Calif.). The flies were kept at 28°C on low-fat powdered dry milk and water, with a 14L:10D photoperiod. Average weight of the flies at the time of experimentation was 0.16 ± 0.02 g.

The American cockroach, *Periplaneta americana* (L.) (Orthoptera: Blattidae), colony was maintained on dog chow (Safeway) and water, at 28°C, 80% humidity, and a 16L:8D photoperiod. Mature females, weighing 1.55 ± 0.06 g, were selected for the experiments.

Swiss-Webster white mice (male) were ordered from Bantin and Kingman Supply (Fremont, Calif.). These animals were kept at 25°C and fed mouse chow (Purina) and water. Average weight of the mice at the time of injection was 27.4 ± 0.6 g.

Mosquito bioassays. Ten *A. aegypti* larvae were placed in a test tube (16 × 125

mm) with 5 ml water (distilled:tap, 1:1), to which was added the BT *israelensis* toxin preparations. Larvae were then incubated at 28°C and mortality was scored at 24 h postinoculation (PI). For adult female *A. aegypti,* test solutions were measured with a microcap and then dispensed into a droplet of mineral oil from which it was taken up into the glass injection tip for delivery. The aliquot containing the test solutions was then introduced into the midgut by rectal injection using a slightly modified system based on that described by Spielman and Wong (27). Hemocoel injections were made using the same caliber glass tip held in a micromanipulator and driven by a water-filled line attached to a micrometer. The tip was inserted in the delicate membrane at the base of the coxa nearest the head. After inoculation, the insects were held at 28°C for 24 hr before mortality was examined.

T. ni bioassays. Toxin preparations were diluted with sterile physiological buffered saline (PBS; 0.15 *M* NaCl, 0.05 *M* Na_2HPO_4, 0.02 *M* KH_2PO_4, pH 7.2) and injected into the larval hemocoel directly in 2- to 3-μl aliquots at the base of the proleg. In feeding experiments, the toxin was suspended in 5% sucrose solution (in water) and fed to the larvae in 2-μl volumes. The larvae were then incubated under regular rearing conditions and examined for mortality at 24 hr PI.

Toxin injections. In vivo toxicity of the alkaline-dissolved BT *israelensis* and BT *kurstaki* toxin to the other test animals was tested by inoculating the toxin, in PBS, directly into the insect hemocoel or intraperitoneally into mice. Mortality was recorded at 24 hr PI.

The mortality data (and results of all BT *israelensis* preparations on larvae of *A. aegypti* and *T. ni*) collected were subjected to Probit analysis (28) to determine the median lethal dose (LD_{50} ± one standard deviation) for each test animal. Four or more concentrations between 10 and 90% mortality, with at least 10 animals per concentration, were performed for each analysis.

RESULTS

Bioassay of the purified BT *israelensis* crystal δ-endotoxin in *A. aegypti* third-stadium larvae gave a LC_{50} of 2.95 ± 0.59 ng/ml. Dissolution of the crystals in a carbonate buffer and bioassay of the protein increased the LC_{50} to 2.29 ± 0.06 μg/ml. A LD_{50} of 54.5 ± 3.1 ppm was obtained when the alkaline-dissolved toxin was administered as an enema to adult female of *A. aegypti.* These numbers are in close agreement with those reported by Klowden *et al.* (29). Besides using bioassay (LD_{50} and LC_{50}) to monitor the purity of the alkaline-dissolved toxins, ELISA procedures specific for BT *israelensis* and BT *kurstaki* crystal proteins were also used to ensure that the materials were not cross contaminated. These ELISA assays are sensitive to the low nanograms/milliliter region; thus, cross-contamination of BT *israelensis* with BT *kurstaki* or the inverse is less than 0.01% by either immunoassay or bioassay. The alkaline-dissolved material, when injected into the body cavity directly, was toxic to all of the animals tested except *T. molitor,* with mice, *T. ni,* and *P. americana* being most sensitive (Table 1, column 2). On the other hand, when challenged orally, none of the animals tested showed sensitivity (at the concentrations tested) toward the alkaline-solubilized toxin except larvae of *A. aegypti* and adults when they were given an enema consisting of the alkaline-dissolved toxin.

When BT *israelensis* was injected at concentrations of 3 to 5 μg/g body wet wt (ppm) into the larvae of *T. ni,* there was a total cessation of heart activity within 30 sec. Hemolymph began to pool in the posterior, as evidenced by an increased green coloration. Wandering activity increased immediately after injection and mouth palpation of the injection site was often observed. Larvae quickly lost motor activity in the

TABLE 1
In Vivo Toxicity of Alkaline-Solubilized BT israelensis and BT kurstaki δ-Endotoxins

Animals	BT *israelensis* LD_{50} (ppm)[a]		BT *kurstaki* LD_{50}	
	Fed	Injected	Fed	Injected
A. aegypti				
Larvae[b]	2.29 ± 0.06 (12.41)[c]	NA[d]	>40	NA
Adults	54.5 ± 3.1 (6.37)	11.6 ± 2.2 (3.16)	>900	>900
T. ni				
Larvae	>50	3.71 ± 0.32 (3.22)	2.30 ± 0.27 (2.85)	>130
H. zea				
Larvae	>35	73.6 ± 3.0 (19.23)	>35	>100
M. domestica				
Adults	NA	10.9 ± 2.2 (2.17)	NA	>150
T. molitor				
Larvae	NA	>100	NA	>100
O. fasciatus				
Adults	NA	27.7 ± 7.0 (1.96)	NA	>300
P. americana				
Adults, female	NA	4.42 ± 0.36 (7.04)	NA	>20
Mouse				
Adults, male	>30	1.31 ± 0.23 (4.47)	>30	>30

[a] Expressed in mg protein per kg body wet weight unless otherwise indicated.
[b] Values are in μg/ml water in which mosquito larvae are incubated.
[c] Values are median lethal dose ± one standard deviation with slope of Probit analyses in parentheses.
[d] NA, data not available.

abdominal region and listed badly when they moved forward. Localized blackening of the body was observed in advanced stages, with subsequent appearance throughout the entire body. At 24 hr PI, failure to respond when the head was mechanically stimulated was used as a criterion for death. Occurrence of paralysis and flaccidity at 30 min PI was always closely correlated with 24-hr mortality.

Direct introduction of alkaline-dissolved toxin into the hemocoel of cockroaches resulted in the loss of motor action immediately. Insects that survived the challenge for 24 hr failed to right themselves, and tremor was observed in all survivors. These effects persisted even when the dosage was as low as 1 ppm.

Intraperitoneal injection of BT *israelensis* into mice at doses close to LD_{50} resulted in lethargy. Mice immediately became ruffled, lost their alertness, and were not inquisitive. Their response to mechanical stimulus was reduced in magnitude, and they were slow in righting themselves. Their breathing appeared shallow. The survivors were severely constipated at 24 hr PI (symptomology was evaluated by Dr. D. Woolley, Department of Animal Physiology, U.C. Davis). At doses equal to or greater than LD_{90}, most of the mice died within 6 hr PI.

Oral administration of the BT *kurstaki* toxin to larvae of *T. ni* resulted in vomitting within 15 min. The larvae stopped feeding thereafter and eventually starved to death. Larvae of *H. zea* were much more resistant (at least 10×) to the BT *kurstaki* toxin than the larvae of *T. ni*. Injection of the dissolved BT *kurstaki* toxin into the body cavity had no adverse effects on any of the animals tested.

SDS–PAGE showed that incubation of BT *kurstaki* and BT *israelensis* crystals in 0.5% Na_2CO_3 (pH 11.0) resulted in solubilization of a number of protein components (Fig. 1, tracks 1 and 2). For BT *israelensis*, these components included three major groups of peptides at 35,000, 27,500 and 23,500 Da (molecular weight determination based on distance of migration in 12.5% SDS–PAGE) and several other minor poly-

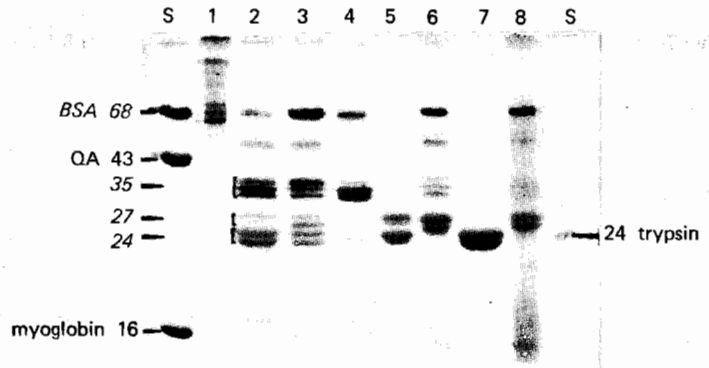

FIG. 1. *SDS–PAGE analysis of alkaline dissolved BT kurstaki and BT israelensis δ-endotoxins. Track 1, BT kurstaki alkaline-soluble crystal protein; track 2, BT israelensis alkaline-soluble crystal protein; track 3, BT israelensis DEAE anion-exchange chromatography effluent; track 4, BT israelensis pH 4.5 insoluble material; track 5, BT israelensis pH 4.5 soluble material; track 6, BT israelensis G-75 effluent, R_f 1.35 fraction; track 7, BT israelensis effluent, R_f 1.53 fraction; track 8, BT israelensis strain IFC-1 alkaline-soluble crystal protein (tracks 1 to 8 have 25 μg protein each); track S, molecular weight (×1000) standard bovine serum albumin (BSA), ovalbumin (OA), trypsin, and myoglobin.*

peptides. These three groups of peptides can be resolved into three, two, and two polypeptides, respectively, by reducing the amount of BT *israelensis* applied. At the same time, the crystal δ-endotoxin of strain IFC-1 was shown to possess the protein components at 27,500- and 27,000-Da as its major constituents (Fig. 1, track 8).

At pH 8.00, a DEAE anion-exchange resin adsorbed all the alkaline-soluble materials from BT *israelensis* δ-endotoxin, and they were recovered by a one-step elution with a salt concentration of 0.15 *M* NaCl (Fig. 1, track 3). When the pH of the effluent from DEAE anion-exchange chromatography was adjusted to 4.5, a precipitate developed quickly while the 23,500- to 27,500-Da polypeptides stayed in solution. Repeated precipitation with the acidic buffer (2×) resulted in a relatively clean preparation of these polypeptides. The precipitate collected from the acidic buffer consisted of the peptides at 35,000-Da plus some of the other minor polypeptides (Fig. 1, tracks 4 and 5). This acid precipitate was insoluble at pH's lower than 10; thus, it was difficult to accurately bioassay against larvae of *T. ni* and *A. aegypti*.

Results of bioassays with several partially purified components of the crystal δ-endotoxin are summarized in Table 2. Neither DEAE chromatography nor acid precipitation (which resulted in the removal of the 35,000-Da polypeptides) led to loss of toxicity. Employing gel filtration with Sephadex G-75, the peptides between 23,500- and 27,500-Da were separated into two fractions with R_f values (V_e/V_o) of 1.35 and 1.53, respectively (Fig. 1, tracks 6 and 7). The R_f 1.35 fraction had the 27,500- and 27,000-Da peptides as its main components. The R_f 1.53 fraction consisted mainly of the 24,000- and 23,500-Da peptides. Toxicity toward larvae of *T. ni* was exhibited by both fractions. The initial neuromuscular symptoms seen with the injection of the alkaline-dissolved crude extract persisted with both fractions. When tested against larvae of *A. aegypti*, only the R_f 1.35 fraction was toxic. A significant drop in toxicity was observed with the R_f 1.53 fraction (Student *t* test, $\alpha = 0.05$, Table 2).

The IFC-1 strain of BT *israelensis* after alkaline dissolution was toxic toward larvae of *A. aegypti*. Also, when injected into larvae of *T. ni*, all the neuromuscular symptoms discussed here were observed.

DISCUSSION

Use of BT *israelensis* as a pesticide for control of mosquitoes and black flies has received considerable attention since the

TABLE 2
Biological Activities of Different Preparations of Alkaline-Solubilized δ-Endotoxin from BT israelensis

Toxin preparations	*A. aegypti* larvae LC_{50} (μg/ml)	*T. ni* larvae LD_{50} (ppm)[a]
Alkaline-dissolved toxin	2.29 ± 0.06 (12.4)[b]	3.71 ± 0.32 (3.22)
DEAE-Cellulose effluent	1.91 ± 0.30 (2.92)	1.96 ± 0.64 (2.16)
pH 4.5 soluble material	2.02 ± 0.54 (2.06)	1.95 ± 0.14 (4.32)
G-75 effluent R_f 1.35	2.71 ± 0.12 (5.72)	3.54 ± 0.50 (3.28)
G-75 effluent R_f 1.53	>20	2.97 ± 0.18 (7.00)
IFC-1 alkaline-dissolved toxin	5.78 ± 0.42 (4.76)	6.09 ± 0.46 (4.67)

[a] Expressed in mg protein per kg body wet weight.
[b] Values are median lethal dose ± one standard deviation, with slope of Probit analysis in parentheses.

recent isolation of the organism (2). The BT *israelensis* toxin is presumed to express its biological effect by causing cytolysis in the host animals. de Barjac (12) first showed that, when BT *israelensis* crystals were ingested, the gut cells of mosquito larvae became swollen and lyzed. Reports by Thomas and Ellar (18, 19) gave biochemical support to the cytolytic properties of BT *israelensis* toxin. To our knowledge, this study is the first to report the *in vivo* neuromuscular effects of BT *israelensis* toxin on an insect host. We have also demonstrated that the toxin is effective against a wider range of animals than previously suspected.

In agreement with other reports, BT *israelensis* crystal δ-endotoxin, when dissolved in alkaline solution, retains its toxicity to both mosquito larvae and adults (29, 30). The most surprising result came when this material was injected directly into the hemocoel of *T. ni* larvae. It led to immediate neuromuscular effects which included listing and heart arrest. *P. americana* lost their motor control immediately after injection and, when they survived the challenge, failed to right themselves and developed tremors. Symptoms observed in mice were very similar to those of botulinum poisoning (a neuromuscular toxin), which include loss of alertness, shallow breathing, and, in some cases, lost activity of the hind legs. Dead animals had a pinched waist, a sign of diaphragm arrest. This by all means does not suggest any similarity between BT *israelensis* and botulinum toxin, but does provide a strong indication that BT *israelensis* δ-endotoxin is exerting neuromuscular effects on the host animals. However, whether these neuromuscular effects are direct actions of the BT *israelensis* toxin or secondary consequences of a primary action of the toxin will need further clarification.

In parallel studies, alkaline-dissolved BT *kurstaki* crystal was shown to differ in several respects from the BT *israelensis* crystal. The symptoms observed following ingestion of BT *kurstaki* were clearly different than those following injection of BT *israelensis*. We did not observe any neuromuscular symptoms when alkaline-dissolved BT *kurstaki* toxin was injected into mice, *T. ni*, *P. americana*, nor any other animals tested. However, neither did we observe any vomitting (an immediate symptom when BT *kurstaki* was fed) when dissolved BT *israelensis* was injected into the larval hemocoel or fed to *T. ni*. The toxin (BT *kurstaki*) fed to the larvae in a single dose (at concentrations greater than 10 ppm) induced vomitting within minutes. At concentrations less than 2 ppm, larvae continued to feed for 15 to 20 min before vomitting began. Significant toxicity of BT *kurstaki* was found only when the toxin was fed to larvae of *T. ni*.

The lower toxicity exhibited following injection of *H. zea*, *T. molitor*, and *O. fasciatus*, as well as when the BT *israelensis* toxin was introduced into the gut of *T. ni*

and adult *A. aegypti,* is physiologically and biochemically very intriguing. The variation in toxicity among the species tested, as in the first case, could mean that the toxin has certain tissue preferences. In the second case, the differences in the route of administration could be interpreted as an adsorption/permeation problem posed by the digestive track of the organism. Bearing in mind the preferential affinity of BT *israelensis* toxin for certain phospholipids (19), an examination of the phospholipid compositions of the different organs (e.g., gut cells vs nerve tissues) may answer some questions about how BT *israelensis* toxin acts *in vivo.*

Partial purification of the toxic element(s) within the crystal δ-endotoxin of BT *israelensis* demonstrated that at least two (or possibly four) polypeptides exhibit different biological activities towards larvae of *A. aegypti* and *T. ni.* The peptides in the R_f 1.53 fraction (24,000- and 23,500-Da) were toxic only to the larvae of *T. ni.* Larvae of *A. aegypti* were not killed by these peptides at concentrations up to 20 μg/ml, 10× that of the LC_{50} for the whole extract. The R_f 1.35 fraction, made up mainly of the 27,500- and 27,000-Da polypeptides, was active against larvae of both *T. ni* and *A. aegypti.* Toxicity was not significantly differnt from the whole alkaline-dissolved toxin (see Table 2). A recent report by Yamamoto *et al.* (32) also gave support to the assignment of the mosquito larvacidal activity to the 27,500- and 27,000-Da polypeptides.

The group of polypeptides at 35,000-Da was separated from the other lower-molecular-weight materials by acid precipitation. However, once precipitated, the 35,000-Da materials could not be kept in solution at a pH lower than 10. This posed serious problems with the bioassay, especially with mosquito larvae which are filter feeders. The presence of a precipitate allowed the larvae to accumulate greater amounts of toxin (that were present as contamination) than they normally would. When a sterile filtrate of the whole alkaline-dissolved toxin was incubated at 15°C for 3 to 4 weeks, a loss of toxicity was accompanied by the loss of the lower-molecular-weight polypeptides. At the end of the incubation period, the major proteins left were 35,000-Da or above in size, and they were not toxic to larvae of *T. ni* nor *A. aegypti.* This may account for the common observation that autoproteolysis is responsible for the loss of toxicity once crystal δ-endotoxin is dissolved, and also suggested that these polypeptides (or at least one of them) are the proteolytic enzyme(s). Furthermore, an antiserum that is active against the 35,000-Da polypeptides failed to react with the toxic materials at 27,500- to 23,500-Da in an indirect ELISA assay. This indicated that the 35,000-Da polypeptides are not only biologically, but also immunologically different from the toxic materials within the crystal δ-endotoxin. Further studies along this line are needed and may prove to be fruitful.

Differences in protein profile of the δ-endotoxin from different strains was reported (31). SDS–PAGE had shown that, of the two strains of BT *israelensis* studied in this report, the IFC-1 is very similar in protein profile to those reported by other investigators (7, 18). The other strain has a far more complex protein composition, consisting of at least two and possibly four peptides that showed different toxic properties. Based on the observations made with these two strains of BT *israelensis,* we postulate that the 27,500-Da polypeptide acts as the native toxic component within the crystal δ-endotoxin. This peptide is toxic toward mosquito larvae and exerts neurotoxic effects once introduced directly into the animals. Removal of small fragments from this polypeptide, probably by autolytic enzyme(s), gives rise to a host of smaller peptides. Depending on how much is hydrolyzed, these smaller peptides may exhibit different host specificities.

In conclusion, the alkaline-dissolved BT *israelensis* δ-endotoxin was shown to be biologically active against a wide spectrum of host animals. *In vivo* neuromuscular effects

of the BT *israelensis* toxin to various insects and a mammal are reported. It was shown that a number of components in the crystal δ-endotoxin (ranging from 27,500- to 23,500-Da) are responsible for the observed neuromuscular symptoms, with the 27,500- and/or 23,500-Da polypeptide as the smallest protein fragment capable of inducing the neurotoxic symptoms in the test animals.

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