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VERTEBRATE TOXICOLOGY OF THE SOLUBILIZED PARASPORAL CRYSTALLINE PROTEINS OF BACILLUS THURINGIENSIS SUBSP. ISRAELENSIS

R. M. Roe, V. L. Kallapur,
W. C. Dauterman and F. W. Edens
Departments of Entomology,
Toxicology, and Poultry Science
North Carolina State University
Raleigh, NC 27695, U.S.A.

M. E. Mayes, G. A. Held and C. Y. Kawanishi
U.S. Environmental Protection Agency
HERL (MD-67)
Research Triangle Park, NC 27711, U.S.A.

A. R. Alford
Department of Entomology
University of Maine
Orono, ME 04469, U.S.A.

C. W. Clifford
Division of Natural Science and Mathematics
Northeastern State University
Tahlequah, OK 74464, U.S.A.

INTRODUCTION

Within the sporangium of the bacterium, Bacillus thuringiensis (Bt), is synthesized a parasporal, proteinaceous crystalline inclusion (1,2) that has found widespread use as a biological control agent (3). Bt is classified into varieties (also called subspecies and serovars) based on the antigenic reactivity of the flagellae (H-antigen) found in young, motile cells (4). As of 1982, cultures representing 28 serovars and 766 different isolates were collected and catalogued (5). The parasporal crystalline proteins (PCP) from these serotypes when ingested have varying degrees of selective toxicity against more than 182 species of insects (3). Research has concentrated on Bt serovar 3a3b, kurstaki (Btk), because of its larvicidal activity against major agricultural pests in the insect order, Lepidoptera (3). The serotype Bt serovar 14, israelensis (Bti), differs from Btk by being toxic to members of the insect order, Diptera (6,7), with little known toxicity to Lepidoptera. Bti is of special interest because of its use in the control of mosquitoes and biting flies.

The anticipated expanded use of Bt in insect control through applications in biotechnology, has stimulated interest in the toxicology of Bt poisoning. Most of the vertebrate toxicological research has focused on Bti. The crystals of Bti consist of several protein components of differing molecular weights. The genes of five polypeptides that compose the Bti parasporal crystal have been cloned. Cry IVA, cry IVB, cry IVC, cry IVD and cytA genes encode polypeptides of 134, 127.8, 77.8, 72.4 and 27.4 K in molecular weight, respectively (8). The cry IVA and B gene products are proteolytically converted to toxic fragments that are in the 58 to 70 K molecular range. Cry IVD protein is proteolytically converted to a fragment of about 30 K. Other polypeptides are also observed as a component of purified crystal but are believed to be degradation products of the major proteins or cellular contaminants (9). The role of the major proteins that make up the parasporal crystal in vertebrate toxicity has been the focus of our research.

TOXICITY OF INJECTED SOLUBILIZED PARASPORAL CRYSTALLINE PROTEIN FROM BT ISRAELENSIS

The alkaline solubilized parasporal crystalline protein (SPCP) of Bti was injected into the hemocoel of insects from 6 orders and intraperitoneally into mice, rats and quail (Table 1). In these studies,

Table 1. Injected toxicity of SPCP.

Animal	24h LD50 (mg/Kg)
Insect[a] (intrahemocoel injections):	
Adults	
Aedes aegypti (yellowfever mosquito)	11.6±2.2
Musca domesticus (housefly)	10.9±2.2
Oncopeltus fasciatus (milkweed bug)	27.7±7.0
Periplaneta americana (American cockroach)	4.42±0.36
Larvae	
Trichoplusia ni (cabbage looper)	3.71±0.32
Heliothis zea (bollworm)	73.6±3.0
Tenebrio molitor (yellow mealworm)	>100
Vertebrates (intraperitoneal injections):	
Swiss-Webster mice	1.31±0.23[a] 2.33[b]
CD rats	1.95 (1.78-2.12)[c]
Japanese quail	22.7 (21.7-24.1)[d]

[a]From Roe *et al.* (10). Values are the mean ± 1 standard deviation.
[b]From Mayes *et al.* (11).
[c]From Mayes, Kallapur, Held, Dauterman, Roe and Kawanishi (unpublished). Value reported is the mean ± the 95% confidence interval (α = 0.05).
[d]From Kallapur, Mayes, Edens, Held, Dauterman, Kawanishi and Roe (unpublished). Value reported is the mean ± the 95% confidence interval (α = 0.05).

Bti was toxic to all of the animals tested except the yellow mealworm. Mice, rats, cabbage loopers, and cockroaches were the most sensitive to SPCP injection with

LD50's of 1.31, 1.95, 3.71 and 4.42 mg/kg, respectively. Among the insects tested, the injected toxicity was not limited to the Diptera (mosquitoes and flies) but was also observed in the Hemiptera, Orthoptera and Lepidoptera. Insect toxicity varied significantly within a single insect family (the Noctuidae) with the LD50 ranging from 3.7 mg/Kg for the cabbage looper to 73.6 mg/Kg for the bollworm. In the vertebrates tested, the lowest LD50 was 1.31 mg/Kg for mice compared to the highest of 22.7 mg/Kg for Japanese quail. In control experiments, Bti SPCP demonstrated typical, oral mosquitocidal activity and was not toxic when fed to Lepidoptera; this was consistent with previous descriptions of the toxic action of Bti (12). These results demonstrate that the solubilized parasporal crystalline protein of Bti is highly toxic and non-specific when introduced by injection into insects and vertebrates.

VERTEBRATE TOXICITY OF BTI SPCP USING DIFFERENT ROUTES OF INTRODUCTION

The toxicity of Bti SPCP introduced by different routes was investigated in the rat, mouse and Japanese quail (Table 2).

Table 2. Toxicity of SPCP by different routes of introduction.

Animal	Route of Introduction	Dose (mg/Kg)	24h % Mortality (n)
CD rat[a]	Intraperitoneal	9	100 (7)
	Subcutaneous	9	0 (12)
	Intravenous	21	0 (6)
	Intratracheal	10	0 (6)
	Gavage (oral)	9	0 (6)
Swiss-Webster mouse[b]	Intraperitoneal	1.4	100 (5)
		1.1	40 (5)
	Gavage (oral)	>30	0 (10)
Japanese quail[c]	Intraperitoneal	30	70 (10)
	Subcutaneous	100	0 (10)
	Intravenous	100	0 (8)
	Intranasal	40	0 (5)

[a]From Mayes, Kallapur, Held, Dauterman, Roe and Kawanishi (unpublished).
[b]From Roe et al. (10).
[c]From Kallapur, Mayes, Edens, Held, Dauterman, Kawanishi and Roe (unpublished).

The only route resulting in death was intraperitoneal injection causing 100% mortality at 9 mg/Kg in rats, 100% mortality at 1.4 mg/Kg and 40% mortality at 1.1 mg/Kg in mice, and 70% mortality at 30 mg/Kg in quail. SPCP was not lethal by subcutaneous, intravenous, intratracheal, and intranasal injection or by gavage. However, subcutaneous injections in rats resulted in localized necrosis relative to dose.

In general Bt has proven to be a very safe insecticide. The route of intoxication responsible for its insecticidal activity in field applications is ingestion. The PCP of Btk ,upon entering the gut of lepidopteran larvae, is activated by the alkaline conditions and proteolytic activity of the digestive system (13-15). The gut epithelial cells swell, vacuoles form, and the cells separate from the basement membrane and each other ultimately disrupting the gut-hemocoel barrier (16-19). Incapacitation and death occurs soon afterward. Similar observations have been made in mosquito larvae fed Bti.

In contrast to insects, no oral toxicity of SPCP in vertebrates was found (Table 2). The fact that the only route of introduction of Bti SPCP that was lethal to vertebrates was intraperitoneal injection and no deaths were noted by other routes, suggests that the target is the peritoneal cavity and its closely associated organs, that interactions occur in this region necessary for toxic action elsewhere, and/or that sequestration or metabolism at other sites of introduction prevent access to the site of action. These possible explanations will be discussed in more detail later.

NEUROTOXIC, MYOTOXIC AND CYTOLYTIC ACTIVITY OF BTI SPCP

The injection of SPCP into insects produced a number of obvious neuromuscular effects almost immediately. These included cardiac arrest, paralysis of the body region near the site of injection, abnormal crawling behavior, and eventual total paralysis within 1 h. Pharmacological studies of ventral nerve cord function in the cabbage looper (Fig. 1) showed that Bti SPCP acted as a nerve poison. Prior to

treatment, ventral nerve cord electrical activity was minimal and a clear response as

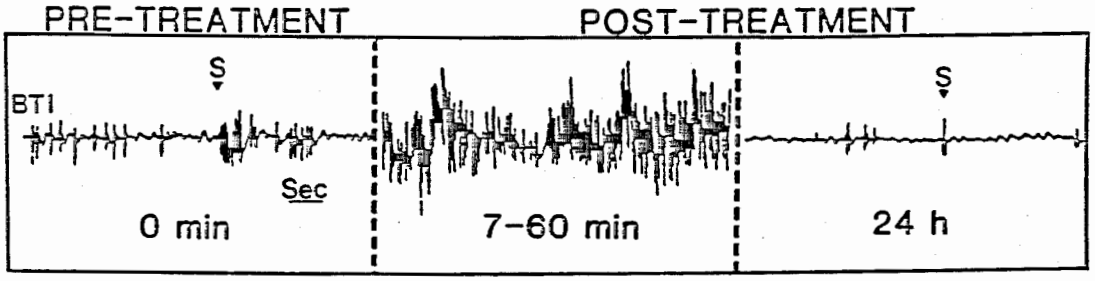

Figure 1. Ventral nerve cord response to injection of 13 mg/Kg of Bti SPCP into the hemocoel of the cabbage looper. In the neuro-physiological preparation, the head, thorax and gut were removed from the larva. Tungsten electrodes were placed into the hemocoel along side abdominal ganglion VIII, the ventral nerve cord and the abdominal wall. Injections of SPCP were made into the second pair of abdominal prolegs. Mechanical sensory stimulation with a glass probe was applied at the anal proleg (indicated as an "S" on the electrical trace of ventral nerve cord activity). Data taken from Roe et al. (10).

indicated by increased electrical activity was noted after stimulation of the insect abdominal prolegs with a glass rod. From 7 to 60 min after the injection of 12 mg/Kg of SPCP, spontaneous-high frequency discharges were recorded in the ventral nerve cord. The insect at this time was completely paralyzed. Hyperexcitability was followed by reduced background activity and sensitivity to sensory stimulation. Similar findings were made in isolated ventral nerve cord and peripheral nerve preparations from the crayfish, Procambarus clarki, treated directly with SPCP (Roe and Grossfeld, unpublished). Neurotoxic and myotoxic activity in insects was also reported by other researchers (20,21).

The behavioral responses of mice to SPCP injection were not as dramatic as that of insects. Mice at 0 to 1 h after injection appeared to be in a stupor and manifested reduced alertness, exploratory behavior, and responsiveness to stimuli. They were also slow in righting themselves due in part to a loss of hind leg control. Some of the dead animals exhibited a constriction at their waist.

The cytolytic activity of Bti SPCP has been well documented. Fig. 2 shows the relationship between SPCP concentration and the percent hemolysis

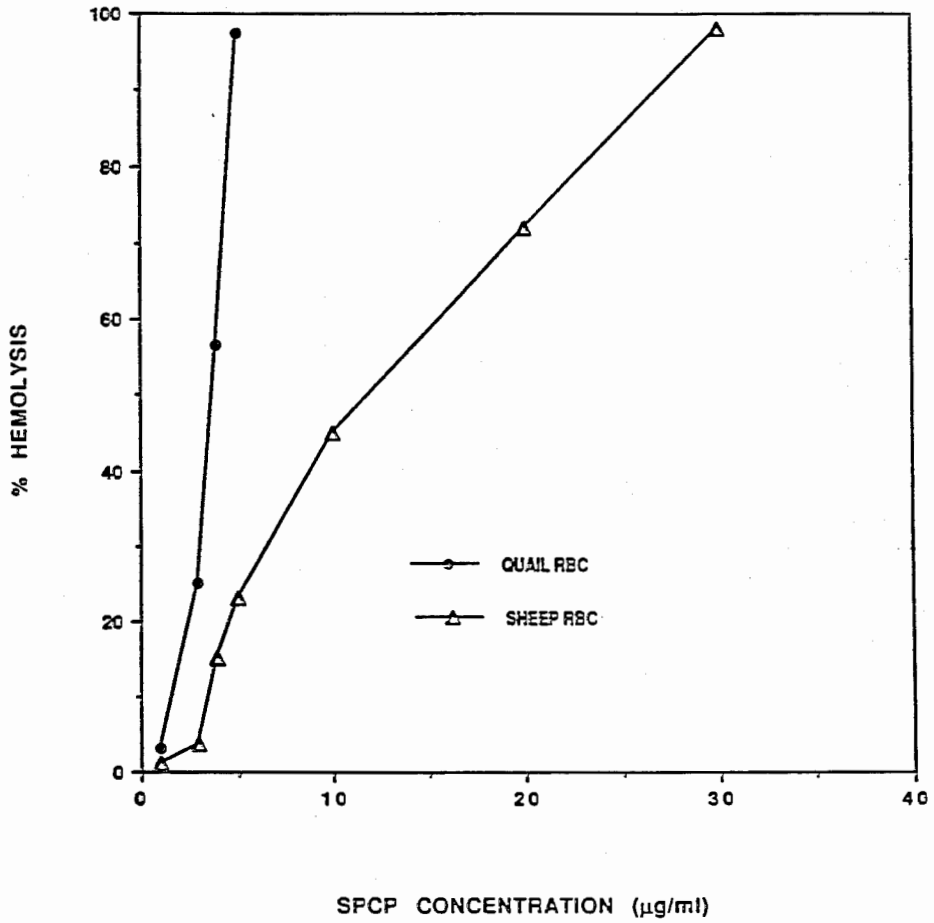

Figure 2. The in vitro hemolytic activity of Bti SPCP (from Kallapur, Mayes, Edens, Held, Dauterman, Kawanishi and Roe, unpublished).

of quail and sheep red blood cells in vitro. It is interesting that this hemolytic activity

is inhibited by preincubation of SPCP with serum. Hemolytic activity has been shown for red blood cells from a number of animals (22) and cytolytic activity for both insect and mammalian cells in culture (23-27). Using the appearance of cytosolic lactate dehydrogenase (LDH) in insect hemolymph after injection of SPCP as a marker for cytolytic activity, Roe et al. (10) reported that SPCP was a potent in vivo cytotoxin in the cabbage looper. However, in vivo cytotoxic activity was not affected by temperature as was the toxicity and onset of hyperexcitability. These results suggested a cause and effect relationship between hyperexcitability and toxicity but not cytotoxicity as measured by LDH levels. Despite the obvious hemolytic activity of Bti SPCP, intraperitoneal injections into mice which produced death in 35 min had no effect on the red blood cell concentration which averaged 11 X 10^6 cells/µl at 0, 20 and 35 min after injection (Roe and Clifford, unpublished). Similar findings were reported by Mayes et al. (11).

TOXIC ACTIVITY OF THE COMPONENTS OF THE BTI PARASPORAL CRYSTAL

Davidson and Yamamoto (28) found that a 25K molecular weight (MW) polypeptide, possibly a fragment of the 28K cytA protein was insecticidal, cytolytic, and lethal to mice. This 25K MW fragment was purified by Sephacryl S-200 gel permeation and DEAE-cellulose chromatography from alkaline dissolved crystal. Similar cytolytic and/or larvicidal properties for 24 to 28K proteins were reported by other researchers (29-32). Contrary to these findings, Hurley et al. (33), Cheung and Hammock (34), Held et al. (35), and Visser et al. (9) found that 25 to 28K proteins were cytolytic but possessed either minimal or no mosquitocidal activity. The purification of Hurley et al. (33) was by gel permeation chromatography on a Bio-Gel P-150 column, Cheung and Hammock (34) by DEAE-Sephacel chromatography, Held et al. (35) by immunoaffinity chromatography, and Visser et al. (9) by sucrose gradient ultracentrifugation. Delécluse et al. (36) most recently found that disruption of the cytA gene eliminates the hemolytic activity of the parasporal crystal.

Ultrastructural studies have revealed that the parasporal body of Bti contains three major inclusion types. Ibarra and Federici (32) isolated the Type 2 inclusion using the pressure of centrifugation to dislodge the inclusion and a NaBr gradient for purification. The Type 2 inclusion which consisted almost exclusively of a 65K protein was only slightly toxic to mosquitoes. Hurley et al. (33, purification previously described) and Kim et al. (37) found that 65K and 67K proteins, respectively, were mosquitocidal. Kim et al. (37) purified the 67K protein by Sepharose CL-4B gel filtration and DEAE-cellulose chromatography from alkaline dissolved PCP. Visser et al. (9) reported mosquitocidal activity and no hemolytic activity for isolated 230 and 130k proteins from Bti. The toxicity of crystalline proteins of Bti derived from recombinant E. coli and Bacillus was recently reviewed by Hofte and Whiteley (8) and Federici et al. (38). There is evidence that deletion of the cytA gene has no effect on the mosquitocidal activity of the parasporal body (36) and others report that cryIVA, B, C and D mosquitocidal activity is enhanced by the presence of cytA (39,40).

In our investigations to assign different toxic activities to cryIV and cytA genes, a 28K protein from Bti (MW determined by SDS-PAGE) was purified and tested for its mosquitocidal, hemolytic, neurotoxic and mouse toxic activities. For the neurotoxicity studies, the 28K protein was purified by DEAE-Sephacel chromatography (41) while for the other tests, the 28K protein was isolated by monoclonal antibody affinity chromatography (11). Table 3 shows that the 28K protein had minimal mosquitocidal

Table 3. The mosquitocidal and hemolytic activity of the components of Bti SPCP[a].

Fraction	Aedes aegypti larval LC50 (μg/ml)[b]	Protein conc. at 50% hemolysis (μg/ml)
Whole SPCP	0.72a	9.3
28K	23.1b	6.1
SPCP less the 28K component	0.81a	no hemolysis at 19.1

[a]From ref. 11.
[b]Means followed by different letters are significantly different by the Tukey's procedure ($\alpha = 0.05$).

activity as compared to both whole solubilized crystal and SPCP without the 28K component. In contrast, the 28K protein had similar hemolytic activity to that of the whole solubilized crystal while the remainder of the crystalline proteins had no effect on hemolysis. The toxic activity in injected mice could also be attributed to the cytolytic 28K component of the solubilized crystal as illustrated in Fig. 3.

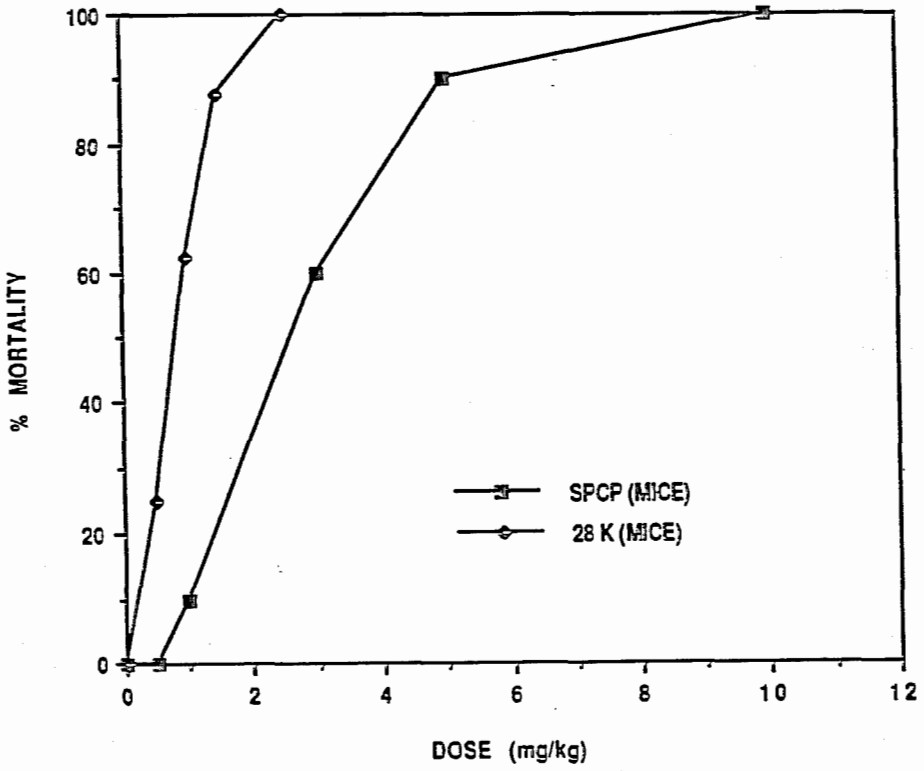

Figure 3. The mouse toxic activity of whole Bti SPCP and the 28K component. Toxins were injected intraperitoneally. The SPCP less the 28K component demonstrated no toxic activity in these studies. Taken from Ref. 11.

The 28K component had comparable activity to that of the whole crystal while SPCP less the 28K protein demonstrated no toxicity in the mouse. In separate studies (10,41), it was discovered that a 25K fragment of the 28K component was also neurotoxic to ventral nerve cord preparations of the cabbage looper causing nerve death in a similar fashion to that of the cytolytic protein, phospholipase A_2. Therefore,

it appears from these studies, that the 28K component has multiple activities that include cytolytic, neurotoxic, and injected mouse toxic activity. However, the 28K protein has low mosquitocidal activity.

MODE OF ACTION OF INJECTED BTI SPCP IN VERTEBRATES

Although the mode of action of Bti SPCP when introduced into vertebrates by intraperitoneal injection is unknown, a number of common physiological responses have been measured both in mice and quail. These include a reduction in heart rate and a decrease in body temperature (Fig. 4). In addition to these effects, peripheral vasodilation is apparent in mice indicated by extreme reddening of the ears, feet and tail. From histopathological studies, the only organs affected by Bti SPCP intraperitoneal injection in mice and rats were the liver and jejunum (11). The jejunum was characterized by hemorrhaging in the lamina propria, especially at the tips of villi and this hemorrhaging was accompanied by epithelial necrosis and cell sloughing. The liver exhibited centrilobular congestion. One explanation for these histopathological responses to Bti SPCP is reduced vascular perfusion possibly resulting from hypotension. The resulting hypoxia would cause cell death. Peripheral circulatory system dilation, decreasing heart rate and temperature, and hypoxia are symptoms consistent with septic shock. The apparent lack of red blood cell hemolysis in vivo in mice dosed with SPCP (discussed earlier) suggests that systemic, general cytolytic activity is not a major contributing toxic factor and that the septic shock-like response is initiated by interactions in the region of the peritoneal cavity. Recall that no other routes of introduction into rats and quail other than by intraperitoneal injection were lethal. The role of cytotoxicity on tissue necrosis in the liver and jejunum and its contribution to the septic shock-like symptoms associated with death is not clear. The localized necrosis due to subcutaneous injections appears to be due to cytolytic activity. Additional references on the vertebrate toxicology of Bt are cited (42-47).

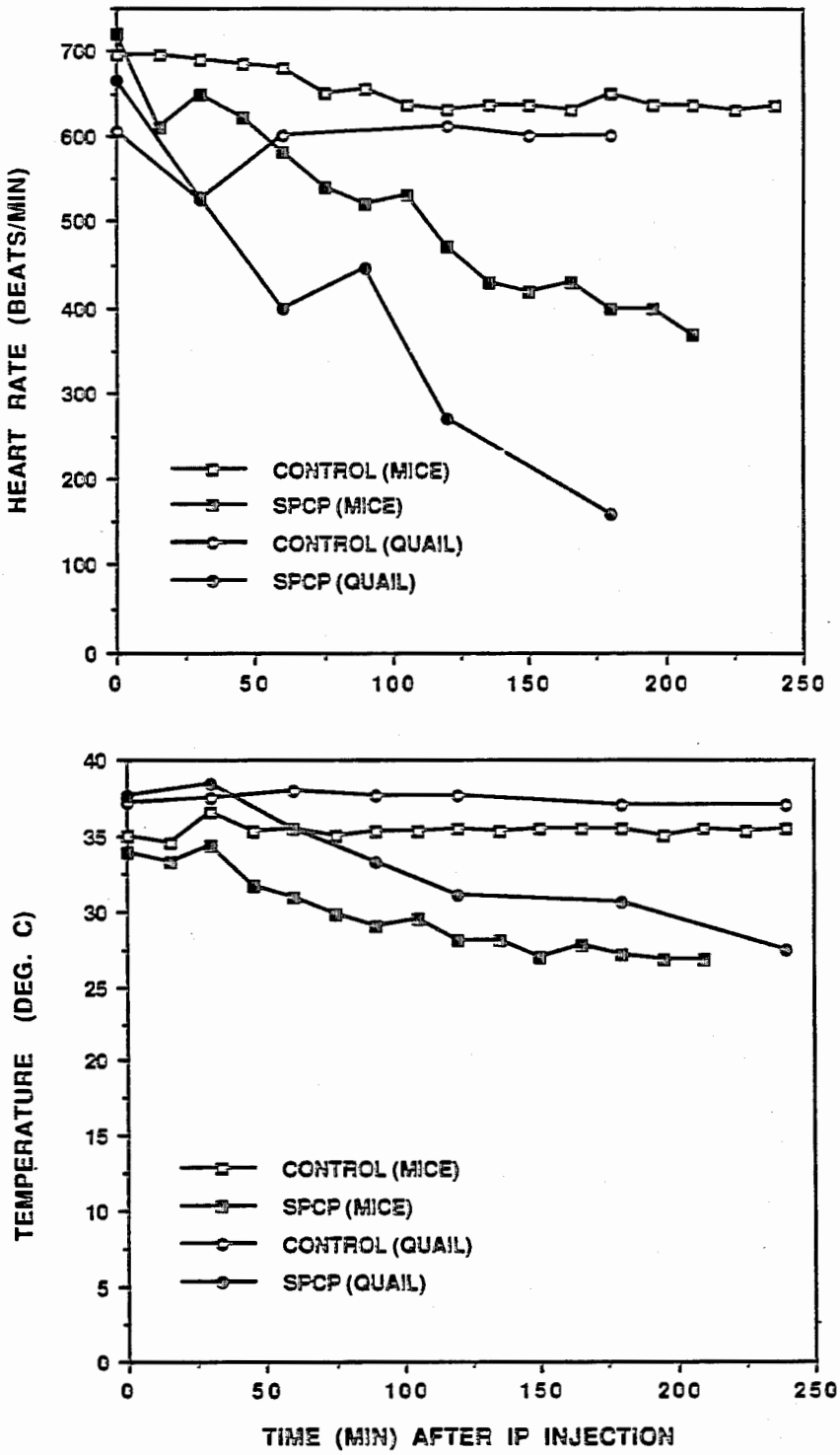

Figure 4. The effect of intraperitoneal injection of Bti SPCP on heart rate and body temperature in mice (11) and Japanese quail (Kallapur, Mayes, Edens, Held, Dauterman, Kawanishi and Roe, unpublished). The SPCP dose was 10 mg/Kg for mice and 30 mg/Kg for quail.

CONCLUSIONS

The solubilized parasporal crystalline protein of Bacillus thuringiensis israelensis has broad-spectrum toxic activity when injected into the hemocoel of insects and into the peritoneal cavity of vertebrates. The mouse LD50 was as low as 1.3 mg of whole SPCP/Kg body weight. SPCP was not lethal when introduced into mice, rats, and quail by other routes including intratracheal, intranasal, intravenous, subcutaneous, and oral administration, demonstrating that Bti is a relatively safe insecticide. Subcutaneous injection does cause localized necrosis. The 28K cytA component of the parasporal crystal is responsible for neurotoxic, hemolytic and mouse toxic activity but has low oral mosquito toxicity. Intraperitoneal injections of SPCP in mice and quail reduced the heart rate and body temperature, produced peripheral vasodilation in mice, and caused jejunal hemorrhaging and liver centrilobular congestion in both mice and rats. These responses are consistent with symptoms associated with endotoxin-induced septic shock.

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16. ABSTRACT

This review summarizes the studies done with the mammalian toxic *Bacillus thuringiensis* subsp. *israelensis* (Bti) 28 kDa cytA protein. The data is relevant to hazard identification studies with bacterial pesticides. The data shows that cytA produces lethal physiological changes in diverse mammalian species when administered intraperitoneally and a dose-dependent localized necrosis by the subcutaneous route. Challenge by other routes have no effects. The cytA protein is a minor component of the insecticidal activity of the Bti parasporal crystal. Insertion of the cytA gene by genetic engineering methods into microbial species that have the potential to invade traumatized tissues or organs could result in detrimental human health effects.

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