

Mechanism of action of *Bacillus thuringiensis var israelensis* insecticidal δ -endotoxin

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Bacillus thuringiensis var israelensis δ -endotoxin protein active against mosquitoes was inactivated by prior incubation with lipids extracted from *Aedes albopictus* cells. Experiments with lipid dispersions and multilamellar liposomes showed that the toxin binds to phosphatidyl choline, sphingomyelin and phosphatidyl ethanolamine provided these lipids contain unsaturated fatty acids. Phosphatidyl serine binds toxin less efficiently and phosphatidyl inositol, cardiolipin, cerebroside and cholesterol show no affinity for the toxin. The results suggest an insecticidal mechanism in which interaction of toxin with specific plasma membrane lipids causes a detergent-like rearrangement of the lipids, leading to disruption of membrane integrity and eventual cytolysis.

<i>Bacillus thuringiensis var israelensis</i>	δ -Endotoxin	Phospholipid receptor	Insecticide
Mosquito	Liposome binding		

1. INTRODUCTION

Certain *Bacillus thuringiensis* strains synthesize intracellular protein inclusions (δ -endotoxins), which are potent insecticides [1] licensed for commercial use on a variety of *Lepidopteran* pests. The toxin from a new serotype H-14, designated *Bacillus thuringiensis var israelensis* is lethal for *Dipteran* larvae [2] including mosquitoes of the genera *Aedes* and *Culex*, which are vectors of malaria and filariasis, respectively. An extensive WHO programme is currently assessing the potential of this serotype for the control of insect vectors of these and other tropical diseases [3].

Despite their importance, the mechanism of action of these toxins is not known. Previous reports have suggested that the primary target of the δ -endotoxin from *B. thuringiensis* serotypes active against *Lepidoptera* is the plasma membrane of gut epithelia and of susceptible cells in vitro [4–12]. In [13] it was demonstrated that, in vivo, the gut epithelium also appears to be a primary target for the *var israelensis* δ -endotoxin. Recently authors in [14] observed that a soluble preparation

of *israelensis* toxin caused rapid cytolysis of insect and mammalian cells in vitro, but had no effect on bacterial protoplasts. This toxin also showed haemolytic activity against a range of erythrocytes. The possibility that the toxin causes lysis directly by interaction with a plasma membrane component was therefore investigated. In this report we identify certain membrane phospholipids as the primary target for the toxin and discuss the mechanism of cytolysis.

Initial experiments showed that the toxin is inactivated by prior incubation with mosquito cell lipids. Using purified lipids we find that the toxin binds readily to multilamellar liposomes containing phosphatidyl choline, sphingomyelin, or phosphatidyl ethanolamine provided these lipids contain unsaturated acyl residues. Phosphatidyl serine binds toxin relatively poorly, but phosphatidyl inositol, cardiolipin, cerebroside and cholesterol show no affinity for the toxin. The results suggest that in susceptible insects the interaction of toxin with specific plasma membrane lipids causes a detergent-like rearrangement of the lipids leading to disruption of membrane integrity

and eventually cytolysis. In this respect the *israelensis* δ -endotoxin may resemble other cytolytic toxins such as *Streptolysin S* [15] in its mode of action.

2. MATERIALS AND METHODS

The sources of strains used [14], growth and sporulation of the microorganisms [16], purification of the crystal δ -endotoxin and the production of the alkali soluble preparations [14] have been described previously.

Suckling mice (average body weight 5 g) were obtained from the Pathology Department, Cambridge University. Bioassays were carried out as in [17]. Growth of *Aedes albopictus* cells in 25 cm² flasks (Nunc) and in vitro toxicity tests were as described in [14].

Phosphatidyl choline (egg), cardiolipin (beef heart) and phosphatidyl serine were obtained from General Biochemicals (Chagrin Falls, OH); cholesterol, dicetylphosphate, sphingomyelin (bovine brain type 1), ethanolamine, α -L-dioleoyl phosphatidyl choline, α -L-dipalmitoyl phosphatidyl choline, α -L-soybean phosphatidyl ethanolamine, α -L-dipalmitoyl phosphatidyl ethanolamine, α -L-dimyristoyl phosphatidyl choline, cerebroside (brain) and stearylamine from Sigma, and phosphatidyl inositol from Koch-Light Laboratories.

2.1. Preparation of liposomes

Liposomes were prepared from chromatographically pure lipids by the following modified [18] procedure: lipid, cholesterol and dicetylphosphate, or stearylamine, were mixed in molar ratios of 2:1.5:0.5, respectively; the total lipid was 35 μ mol. The mixture was dried as a thin film in vacuo in a 100 ml round-bottomed flask and then resuspended with gentle hand shaking in 3 ml of phosphate buffered saline (PBS). Further dispersion of the lipids was accomplished by brief treatment (2–5 min) in a sonic bath.

2.2. Extraction of cell lipids

Lipids were extracted from *A. albopictus* cells grown in 25-cm² flasks and *B. megaterium* KM vegetative cells by a modified Folch [19] procedure.

2.3. Analytical

Neutralisation of the alkali-soluble crystal δ -endotoxin was detected by measuring the potency of the toxin after incubation with liposomes for 2 h at 0, 4, 20, 37 or 44°C (toxin–lipid ratio of 1:10 (w/w), except where stated). The supernatants remaining after centrifugation of toxin–liposome mixtures at 50000 \times g for 10 min were also tested for toxicity. Toxicity of the soluble δ -endotoxin was measured in vivo by bioassay of *A. aegypti* 3rd instar larvae [17] and by subcutaneous inoculation of suckling mice, and in vitro by observation of cytopathic effects on *A. albopictus* cells [14]. Control experiments were run in parallel with alkali soluble δ -endotoxin, or liposome preparations alone.

3. RESULTS AND DISCUSSION

In preliminary screening experiments to identify putative toxin receptors on mosquito cells it was found that the toxin was effectively neutralised by preincubation with a 10-fold excess of a sonicated dispersion of phospholipids purified from cultured *Aedes albopictus* cells.

These results raised the possibility that plasma membrane phospholipids may be a target for the cytopathic action of the δ -endotoxin. Other cytolytic toxins have been reported to interact with phospholipids either in a detergent-like manner, or by activating endogenous membrane phospholipases [15,20–27].

As table 1 shows, preincubation with the mosquito phospholipids neutralised both in vitro and in vivo toxicity in comparison to untreated controls. In control experiments with δ -endotoxin preincubated with PBS alone, 5 μ g of δ -endotoxin.ml⁻¹ of tissue culture medium caused total lysis of *A. albopictus* cells in the in vitro assay [14]. This δ -endotoxin, preincubated with PBS also caused 100% mortality when inoculated subcutaneously into suckling mice at a concentration of 25 μ g.g⁻¹ body weight [14]. Lipid dispersions prepared from the Gram-positive bacterium *Bacillus megaterium* KM failed to neutralise the toxin even at toxin–lipid ratios of 1:50 (w/w).

In contrast to mosquito cells, *Bacillus megaterium* KM membranes contain no phosphatidyl choline, sphingomyelin, cholesterol or significant amounts of unsaturated fatty acids

[27]. This suggested that the specificity of toxin-lipid interaction may be determined by one or more membrane components. A series of experiments was therefore carried out in which sonicated dispersions of purified lipids with varying composition were tested for their ability to neutralise the δ -endotoxin.

The results (table 1; fig.1) show that preincubation with sonicated dispersions prepared solely from phosphatidyl choline is sufficient to neutralise the toxin in vitro and in vivo. In an attempt to reproduce more closely the lipid structure found in the plasma membrane of susceptible cells, multilamellar liposomes were prepared by addition

Table 1

Effect of various lipid preparations on toxin action in vivo and in vitro

Liposome composition ^a	Toxin:lipid ratio (w/w)	Cytopathic ^b effect	In vivo toxicity ^c number dead/number used
<i>A. albopictus</i> cell lipid	1:10	None	0/9
<i>B. megaterium</i> KM cell lipid	1:50	Lysis	—
Phosphatidyl choline	1:10	None	0/2
Phosphatidyl choline:cholesterol:stearylamine	1:10	None	0/11
Phosphatidyl choline:cholesterol:dicetylphosphate	1:10	None	0/11
Sphingomyelin:cholesterol:stearylamine	1:10	None	0/11
Sphingomyelin:cholesterol:dicetylphosphate	0:10	None	0/8
Soybean phosphatidyl ethanolamine:cholesterol:dicetyl phosphate	0:10	None	0/10
Phosphatidyl serine:cholesterol:dicetyl phosphate	1:10	Lysis	—
Phosphatidyl serine:cholesterol:dicetyl phosphate	1:25	None	—
Cardiolipin:cholesterol:dicetyl phosphate	1:25	Lysis	20/20
Phosphatidyl inositol:cholesterol:dicetyl phosphate	1:25	Lysis	4/4
Cerebroside:cholesterol:dicetyl phosphate	1:50	Lysis	—

^a Molar ratios were 2:1.5:0.5. Preparation, dispersion and sonication of all lipid and liposome preparations was carried out at 30°C except in the case of cerebroside containing liposomes which were prepared at 60°C and subsequently assayed at 20°C

^b Cytopathology observed after exposure of *A. albopictus* cells to a solution of 5 μ g δ -endotoxin/ml after it has been incubated with the appropriate lipid

^c Subcutaneous inoculation of suckling mice; an equivalent of 25 μ g δ -endotoxin/g mouse was used

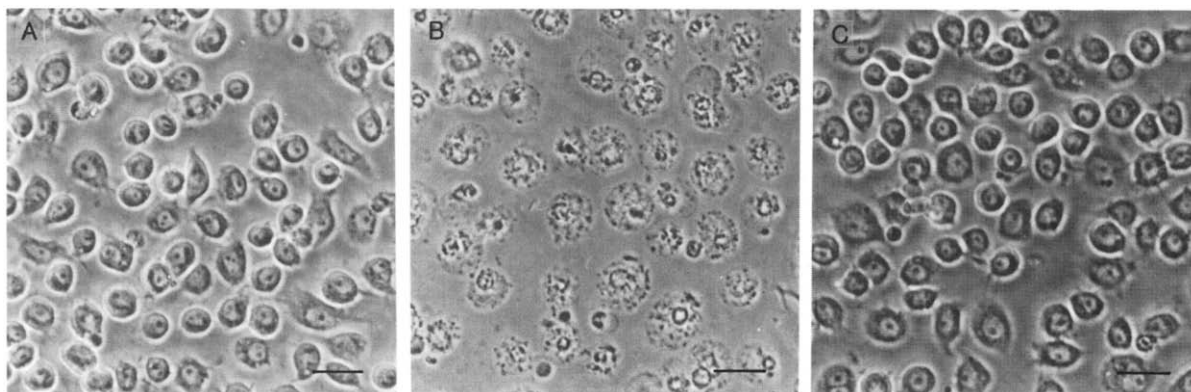


Fig.1. Phase-contrast light micrographs of *A. albopictus* cells treated with: (A) egg phosphatidyl choline dispersions for 30 min as control; (B) 5 μ g alkali-soluble *israelensis* toxic protein/ml for 20 min; (C) 5 μ g alkali-soluble *israelensis* toxic protein/ml preincubated with egg phosphatidyl choline dispersions 1:10 (w/w) for 2 h at 37°C (bars, 25 μ m).

of cholesterol to phosphatidyl choline and inclusion of either stearylamine or dicetylphosphate, to confer, respectively, a net positive or negative charge on the lipid bilayer [28]. Table 1 shows that liposomes containing phosphatidyl choline and cholesterol neutralised the toxin at least as effectively as sonicated phosphatidyl choline dispersions. Thus the presence of the sterol does not appear to be essential for toxin neutralisation and this was confirmed by experiments in which aqueous cholesterol dispersions [29] were found not to inhibit toxin action (not shown).

The net charge on the liposomes did not appear to be a determining factor in toxin-lipid interaction since no difference was observed (table 1) between phosphatidyl choline liposomes containing either stearylamine, or dicetylphosphate.

Preliminary experiments to investigate the influence of the phospholipid headgroup are also shown in table 1. Sphingomyelin and phosphatidyl ethanolamine were as effective as phosphatidyl choline and at higher toxin/lipid ratios, phosphatidyl serine showed some neutralising ability. All other phospholipids tested proved inef-

fective in vitro and in vivo even at high toxin/lipid ratios. Liposomes prepared from cerebroside were also unable to neutralise the toxin.

A mechanism for toxin neutralisation based on binding to susceptible liposomes appeared the most likely explanation of the above results. This was confirmed by the finding that supernatants obtained after centrifugation of egg phosphatidyl choline-toxin incubations were devoid of toxin activity (not shown). This result was obtained when the incubations were performed at 0, 20, 37 or 44°C.

To examine the influence of the fatty acid moieties of the phospholipids on toxin-lipid interaction, liposomes were prepared from egg lecithin and from various synthetic phosphatidyl cholines and phosphatidyl ethanolamines (table 2). The importance of the physical state of the lipid bilayer was also investigated by carrying out the liposome preparation, and toxin preincubation at defined temperatures chosen with reference to the chain melting temperatures of the phospholipids.

Table 2 shows that liposomes prepared from egg phosphatidyl choline with, or without cholesterol

Table 2

Toxin neutralisation: influence of liposome composition and temperature

Liposome composition ^a	Toxin:lipid ratio (w/w)	Incubation temp. (°C)	Cytopathic ^b effect
Phosphatidyl choline (egg):cholesterol:dicetyl phosphate (20°C)	1:10	0, 20, 37 or 44	None
Phosphatidyl choline (egg):dicetyl phosphate (20°C)	1:10	0, 20, 37 or 44	None
Dioleoyl phosphatidyl choline:cholesterol:dicetyl phosphate (20°C)	1:10	20, 37 or 44	None
Dioleoyl phosphatidyl choline:stearylamine (20°C)	1:10	20, 37 or 44	Lysis
Dipalmitoyl phosphatidyl choline:cholesterol:dicetyl phosphate (45°C)	1:20	20, 37 or 44	Lysis
Dipalmitoyl phosphatidyl choline:stearylamine (45°C)	1:20	20, 37 or 44	Lysis
Dimyristoyl phosphatidyl choline:cholesterol:dicetyl phosphate (35°C)	1:50	4, 20 or 37	Lysis
Soybean phosphatidyl ethanolamine:cholesterol:dicetyl phosphate (20°C)	1:10	20 or 37	None
Dipalmitoyl phosphatidyl ethanolamine:cholesterol:dicetyl phosphate (45°C)	1:50	20 or 37	Lysis

^a Molar ratios were 2:1.5:0.5. Preparation, dispersion and sonication of each liposome preparation was carried out at the temperatures in parentheses

^b Cytopathology observed after exposure of *A. albopictus* cells to a solution of 5 µg δ-endotoxin/ml after it has been incubated with the appropriate phospholipid

were equally able to bind toxin at all 4 test temperatures. Since the chain melting transition of egg phosphatidyl choline occurs between -7 and -15°C [30], the hydrophobic region of the lipid bilayer will be in the liquid crystalline, or fluid state in these liposomes. Dioleoyl phosphatidyl choline has a chain melting temperature of -22°C and would therefore also be in the fluid state above 0°C . Liposomes composed of this lipid plus cholesterol were able to bind the toxin at 20, 37 or 44°C (table 2).

In contrast to the above results with phosphatidyl choline containing unsaturated fatty acids, table 2 shows that liposomes prepared from the synthetic saturated dipalmitoyl (C16:0) and dimyristoyl (C14:0) phosphatidyl cholines plus or minus cholesterol, were unable to bind and hence neutralise the toxin at any of the test temperatures at lipid/toxin ratios of 1:20 and 1:50 (w/w).

The ability of the unsaturated phosphatidyl cholines to bind toxin could indicate a specific requirement for unsaturated hydrocarbon chains for toxin-lipid association, or the need for a fluid bilayer, or both. The experiments with the saturated phosphatidyl cholines enable us to distinguish between these possibilities, however, as follows: The chain melting temperature of dimyristoyl phosphatidyl choline is 23°C [15]. Thus at 37°C , liposomes composed of this lipid plus cholesterol are in the fluid state, whereas at 4°C the liposomes would be in the more rigid gel state. Table 2 shows, however, that these liposomes were unable to bind the toxin at all test temperatures including 37°C . By analogy with similar experiments on the *Streptolysin S* toxin [15], if bilayer fluidity were the sole factor in the hydrophobic region influencing toxin interaction, dimyristoyl phosphatidyl choline liposomes would be expected to bind the toxin at 37°C , but not at 3°C .

Additional evidence that bilayer fluidity per se is not sufficient for phosphatidyl choline-toxin interaction is provided by the experiments with dipalmitoyl phosphatidyl choline in table 2. Pure preparations of this phospholipid show a gel-fluid transition at 42°C [31] and this is likely to be broadened by the inclusion of cholesterol in the liposomes. Even at incubation temperatures as high as 44°C , however, the dipalmitoyl phosphatidyl choline liposomes were unable to

bind the toxin.

With respect to interaction of the toxin with the hydrophobic portion of the lipid bilayer, the above results strongly suggest a specific role for unsaturated fatty acids in addition to a need for a fluid bilayer. This suggestion is reinforced by the experiments with phosphatidyl ethanolamine and dioleoyl phosphatidyl choline in table 2. Liposomes containing highly unsaturated (primarily C18:2, linoleic acid) soybean phosphatidyl ethanolamine bound the toxin very effectively at 20 and 37°C , in contrast to the saturated dipalmitoyl phosphatidyl ethanolamine liposomes which would also be in the fluid state at 37°C because of the additional presence of cholesterol.

When cholesterol was omitted from the dioleoyl phosphatidyl choline liposomes they no longer bound the toxin at 37 or 44°C (table 2). As previously suggested in [32] and in [33], we interpret this result to indicate that above 20°C , dioleoyl phosphatidyl choline dispersions lacking cholesterol become increasingly disordered and unable to assume the stable bilayer structure required for toxin interaction. Addition of cholesterol serves to tighten the bilayer and thereby broaden the temperature range over which dioleoyl phosphatidyl choline can form a stable bilayer [34]. Consequently the sterol containing liposomes can bind toxin at temperatures as high as 44°C .

In addition to demonstrating a requirement for unsaturated fatty acids contained within a fluid bilayer the experiments in table 1 and 2 reveal a third specificity determinant located in the polar portion of the phospholipids. Thus, although the experiments indicate that net surface charge is not a crucial determinant, the results with the different phospholipids suggest that liposomes containing either the zwitterionic phosphoryl choline group or phosphatidyl ethanolamine bind the toxin much more effectively than liposomes containing the anionic phosphatidyl serine. No toxin binding occurs to liposomes containing either strongly acid headgroups (cardiolipin or phosphatidyl inositol) or cerebroside.

The result with cardiolipin (beef heart) liposomes is particularly informative since this phospholipid contains primarily unsaturated (C18:1, C18:2) fatty acids [35] and when combined with cholesterol in liposomes is likely to form a lamellar fluid bilayer at 20°C . The fact that these

liposomes possess a hydrophobic phase containing the two specificity determinants of unsaturation and fluidity and yet are unable to bind the toxin, emphasises the key role of the phospholipid polar group in bilayer-toxin interaction.

In summary, these data suggest that phosphatidyl choline, sphingomyelin, phosphatidyl ethanolamine and to a lesser extent phosphatidyl serine are the cellular targets of the δ -endotoxin, provided these lipids contain unsaturated acyl residues. The *in vitro* experiments also point to a need for an organised fluid bilayer for toxin binding.

In other experiments (not shown) the toxin was observed to cause a decrease in the turbidity of liposome suspensions, suggesting that smaller lipid structures are formed upon interaction with toxin. This suggestion was confirmed by electron microscopy of negatively stained toxin-liposome preparations which showed blebbing of the multilamellar vesicles similar to that observed previously with insect cells [14].

In many respects therefore the *israelensis* δ -endotoxin resembles a number of other cytolytic toxins which bind specifically to membrane lipid components and cause lysis by perturbing and rearranging the lipid bilayer. In some instances [36] membrane cholesterol is the primary target, while others [15,20,21,23-25] resemble the *israelensis* toxin in binding specifically to phospholipids. A number of these cytolytic toxins have been reported to stimulate endogenous phospholipase activity in the membrane of susceptible cells [23,37] leading to the suggestion that cytolysis results indirectly from the damaging effects of the resultant lysolipids and fatty acids. No such stimulation was observed when *Aedes albopictus* cells labelled by biosynthetic incorporation of lipid precursors [37] were exposed to the *israelensis* toxin (not shown), and we conclude that like *Streptolysin S* and *Staphylococcus* α toxin [15,23], this toxin acts directly as a protein-surfactant to destroy plasma membrane integrity.

These results do not immediately suggest an explanation for the insensitivity of other insect species to the toxin; e.g., *Lepidoptera*, compared to *Diptera*. Although *Lepidopteran* larvae are insensitive when fed the toxin orally [14], they succumb readily to toxin injected into the haemocoel (not shown). Further experiments are in progress

to investigate the importance of other factors such as gut conditions and the chemistry of gut epithelial cell surfaces in determining insecticidal potency and specificity.

All the above experiments were carried out using the alkali-solubilized toxin described in [14]. However, recent work (to be published elsewhere) has shown that the M_r 28000 component of this soluble preparation is the polypeptide responsible for the cytolytic activity of this δ -endotoxin.

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