Lectin-like binding of *Bacillus thuringiensis* var. *kurstaki* lepidopteran-specific toxin is an initial step in insecticidal action

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Received 1 February 1984

The two δ-endotoxins comprising the *Bacillus thuringiensis* var. *kurstaki* HD1 insecticidal protein crystal were separated. The lepidopteran-specific protoxin was activated in vitro and its mechanism of action investigated. Toxicity towards *Choristoneura fumiferana* CF1 cells was specifically inhibited by preincubation of the toxin with *N*-acetylgalactosamine and *N*-acetylneuraminic acid. The lectins soybean agglutinin and wheat germ agglutinin, which bind *N*-acetylgalactosamine, also inhibited toxicity. Since *N*-acetylneuraminic acid is not known to occur in insects, these results suggest that the toxin may recognise a specific plasma membrane glycoconjugate receptor with a terminal *N*-acetylgalactosamine residue.

Bacillus thuringiensis *var.* kurstaki *δ-Endotoxin* *Insecticide* *Lectin* N-*Acetylgalactosamine* *Glycoconjugate receptor*

1. INTRODUCTION

The crystalline protein δ-endotoxin of *Bacillus thuringiensis* var. *kurstaki* is toxic to the larvae of many lepidopteran species. Although it has been used as a commercial insecticide for 20 years the molecular basis for its insect toxicity has not yet been identified.

Native crystal δ-endotoxin is a water-insoluble protoxin which is solubilised in the alkaline conditions of the larval gut and activated by gut proteases. In vitro activation of the δ-endotoxin has been achieved by incubation of the crystals at high pH under reducing conditions and with insect gut proteases [1,2]. Histological studies in vivo have shown the primary target to be the larval midgut epithelial cells which swell and lyse causing severe disruption of the gut wall [3–6]. In vitro the activated toxin causes cytolysis of certain lepidopteran cell lines and larval midgut cells [7–9]. Cytological effects have been reported as early as 1 min after exposure to the toxin [6,10]; susceptible cells exhibit a rapid general breakdown of permeability barriers to small ions, dyes and internal markers [4,8,9].

The crystal δ-endotoxin of the HD-1 strain of *B. thuringiensis* var. *kurstaki* has two distinct toxic moieties, a lepidopteran-specific toxin and a 'mosquito factor' toxic to both mosquito and lepidopteran larvae [11]. These can be separated under alkaline and reducing conditions [12]. The work described here refers only to the lepidopteran-specific toxin. The narrow spectrum of activity of this toxin and its speed of action suggest that it acts directly on a specific receptor present only in the plasma membrane of susceptible lepidopteran cells.

Plasma membrane receptors for the potent mosquitocidal *Bacillus thuringiensis* var. *israelensis* δ-endotoxin have been identified as phosphatidylcholine, sphingomyelin, phosphatidylethanolamine and phosphatidylserine [13]. The ubiquitous presence of these phospholipids in eukaryotic membranes explains the finding that *B. thuringiensis* var. *israelensis* δ-endotoxin activated in vitro is cytolytic to a wide variety of cell types including mammalian cells [12]. In contrast, the observation that even after activation the *B. thuringiensis* var. *kurstaki* δ-endotoxin affects only lepidopteran larvae and cell lines suggests that the membrane

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00145793/84/$3.00

receptor for this toxin may be cell specific either in whole or in part.

The identity of this proposed specific plasma membrane receptor has been investigated by assessing the ability of various molecules to neutralise the cytolytic effect of activated lepidopteran-specific toxin against *Choristoneura fumiferana* CF1 cells in vitro.

2. MATERIALS AND METHODS

The source of *B. thuringiensis* var. *kurstaki* HD1 [12], growth and sporulation of the microorganism [14], purification of the crystal δ-endotoxin and the production of alkali-soluble preparations [12] have been described.

Pieris brassicae larvae and suckling mice were obtained as in [12]. In vivo assays, growth of *C. fumiferana* CF1 cells and in vitro toxicity tests were as in [12]. Human erythrocytes were obtained from W. Thomas. Haemolytic activity was assayed as in [12]. Cell lipid extraction [15] and liposome preparation [13] have been described. All monosaccharides and lectins were obtained from Sigma, except concanavalin A from Miles-Yeda.

2.1. *Preparation of larval gut extract*

Midguts from 12 *P. brassicae* 4th instar larvae were excised, homogenised in 1 ml 50 mM Na_2CO_3–HCl (pH 9.5) containing 10 mM dithiothreitol (DTT), and the homogenate centrifuged at 10000 × *g* for 5 min. The soluble gut extract was then filtered aseptically through a 0.45 μm Millipore filter. The filtrate was used immediately or stored frozen at −20°C.

2.2. *Preparation of activated lepidopteran toxin*

B. thuringiensis var. *kurstaki* HD1 crystal δ-endotoxin was solubilised [12] and the insoluble mosquito factor [11] removed by centrifugation as in [12]. The soluble lepidopteran-specific component was activated either by incubation at 20°C for a minimum of 7 days, or by incubation with *P. brassicae* gut extract. The alkali-soluble fraction (1 mg protein · ml^{-1}) was incubated with gut extract (4:1, v/v) at 37°C for 15 min, then mixed (5:1, v/v) with foetal calf serum (FCS) prior to addition to the cells.

2.3. *Analytical*

Protein estimation was as in [16] using bovine serum albumin as a standard. SDS-polyacrylamide gel electrophoresis was conducted as in [12]. *N*-Acetylneuraminic acid was assayed by the thiobarbituric acid method [17] using cells washed 3 times in phosphate-buffered saline (PBS) with subsequent hydrolysis at 80°C for 60 min in 0.1 N H_2SO_4.

3. RESULTS AND DISCUSSION

Separation of the insoluble 63 kDa mosquito factor from the alkali-soluble lepidopteran-specific toxin as in [12] is shown in fig.1, tracks 5 and 6. Both methods of activation (see section 2.2) of the soluble toxin yielded a major polypeptide of 54 kDa with minor components of lower molecular mass. The degradation of the soluble toxin over several days at 20°C is caused by crystal-associated proteases [18,19]. This proteolysis is essentially complete after 10 days and the polypeptide profile remains stable for at least 60 days at 20°C (fig.1, tracks 7–10).

It seems likely that the 54 kDa polypeptide is analogous to the 59 kDa polypeptide produced by treatment of alkali-solubilised δ-endotoxin with *Bombyx mori* gut extract [20]. This 59 kDa polypeptide was shown to be the primary toxic principle responsible for toxicity towards lepidopteran larvae [20]. Incubation of soluble toxin with *P. brassicae* gut extract at 37°C for longer than 30 min resulted in the appearance of a 50 kDa polypeptide concomitant with a decrease in the intensity of the 54 kDa band and a reduction in in vitro toxicity to CF1 cells (not shown).

When activated by either procedure the soluble toxin retained its lepidopteran specificity. Thus the activated toxin was lethal to *P. brassicae* larvae in vivo, but no toxic effects were observed on injection of suckling mice (table 1). Similarly activated toxin at 50 μg · ml^{-1} caused 50% lysis of CF1 cells in 60 min in vitro, as assessed by vital staining with trypan blue, but human erythrocytes were unaffected by 100 μg · ml^{-1} toxin after 3 h. Controls containing 50 mM Na_2CO_3–HCl (pH 9.5) and 10 mM DTT, with *P. brassicae* gut extract and FCS where appropriate, showed no toxic effects in vivo or in vitro.

Preincubation with various lipid preparations

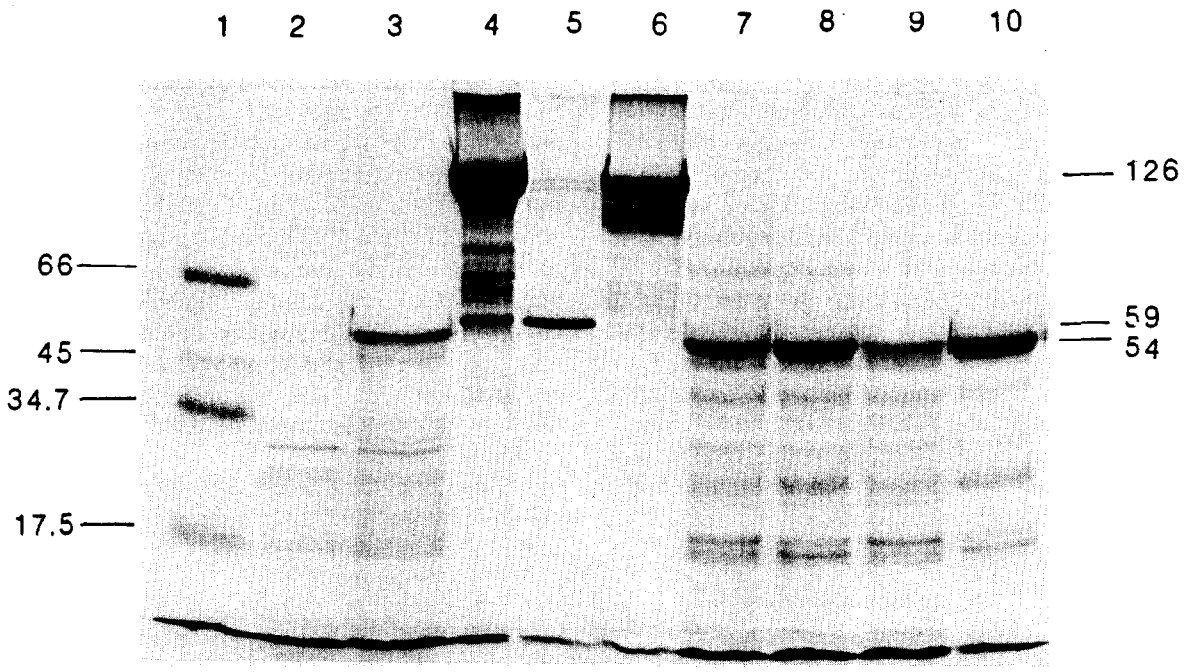

Fig.1. SDS/10% polyacrylamide gel electrophoresis, Coomassie blue-stained. Track 1, molecular mass standards (kDa): 50 μg albumin (66), ovalbumin (45), pepsin (34.7), lactoglobulin (17.5); track 2, 10 μl *P. brassicae* gut extract; track 3, 50 μg soluble crystal protein incubated with 10 μl *P. brassicae* gut extract for 15 min, 37°C; track 4, 50 μg native crystal δ-endotoxin; track 5, insoluble crystal protein and track 6, soluble crystal protein obtained by incubation of 50 μg native crystal δ-endotoxin in 50 mM Na_2CO_3–HCl (pH 9.5), 10 mM DTT; track 7, 50 μg soluble crystal protein after 20 days, 20°C; track 8, 50 μg soluble crystal protein after 33 days, 20°C; track 9, 50 μg soluble crystal protein after 45 days, 20°C; track 10, 50 μg soluble crystal protein after 62 days, 20°C.

Table 1

In vivo assays

Test sample	Amount per animal	Test animal[b]	Method of administration[c]	No. dead/ total no.
Active soluble toxin in	10 μg	suckling mice	s.c.	0/6
50 mM Na_2CO_3–HCl (pH 9.5)	2 μg	*P. brassicae* larvae	i.h.	10/10
+ 10 mM DTT[a]	2 μg	*P. brassicae* larvae	per os	10/10
50 mM Na_2CO_3–HCl (pH 9.5)	50 μl	suckling mice	s.c.	0/6
+ 10 mM DTT	2 μl	*P. brassicae* larvae	i.h.	0/10
	2 μl	*P. brassicae* larvae	per os	0/10

[a] Soluble toxin was activated by incubation at 20°C for 14 days

[b] White Balb/c mice (5 days old) and 4th instar *P. brassicae*

[c] s.c., subcutaneous inoculation into right thigh; i.h., intrahaemocoel injection; per os, force fed larvae

was used to investigate the possibility that the lepidopteran-specific *B. thuringiensis* var. *kurstaki* toxin caused cytolysis by interaction with plasma membrane phospholipids as observed for the *B. thuringiensis* var. *israelensis* mosquitocidal δ-endotoxin [13]. The inability of the lipid preparations used (table 2) to neutralise toxicity against CF1 cells suggested that the specificity of toxin–membrane interactions was determined by a different membrane component. A series of experiments was carried out to test the ability of various lectins and monosaccharides to neutralise the toxin. Initial experiments (table 3) indicated that the toxin was completely inactivated by prior incubation with *N*-acetylgalactosamine (GalNAc) and *N*-acetylneuraminic acid (NeuNAc), but not by a range of other monosaccharides tested. In particular the inability of *N*-acetylglucosamine (GlcNAc) to neutralise toxicity precludes a non-specific effect.

Of the lectins tested for toxin-neutralising ability only wheat germ agglutinin (WGA) and soybean agglutinin (SBA) gave partial protection of CF1 cells from the toxin (table 4). This effect was noted both with prior incubation of cells with lectin, and of activated toxin with lectin. The ability of WGA to inhibit toxicity was abolished when the lectin was incubated with 250 mM GlcNAc prior to incubation with cells or toxin as described above. It was observed that SBA ($50\ \mu g \cdot ml^{-1}$) caused agglutination of CF1 cells, indicating the presence of GalNAc as a terminal residue on glycoconjugates on the cell surface.

No insects so far tested have contained NeuNAc [21–23]. When CF1 cells were assayed for sialic acid, the amount detected was less than

Table 2
Effect of preincubation with lipids on toxicity of soluble δ-endotoxin in vitro

Lipid or liposome composition[a]	Toxin : lipid ratio	Incubation temperature[b] (°C)	Cytopathic effect[c]
Aedes albopictus cell lipid	1:10	20	lysis
Choristoneura fumiferana cell lipid	1:25	20	lysis
Pieris brassicae 4th instar larval gut lipid	1:25	20 37	lysis
Brain cerebroside: cholesterol: dicetyl phosphate	1:25	20 37	lysis
Egg phosphatidylcholine: cholesterol: dicetyl phosphate	1:10	20	lysis
Cardiolipin: cholesterol: dicetyl phosphate	1:10	20	lysis
Cholesterol	1:25	20 37	lysis

[a] Liposome molar ratios were 2:1.5:0.5
[b] Lipid and activated soluble δ-endotoxin were incubated together for 120 min before addition to cells at 20°C
[c] Cytopathology observed in *C. fumiferana* cells at toxin concentration of $50\ \mu g \cdot ml^{-1}$

Table 3

Effect of preincubation with monosaccharides on toxicity of soluble δ-endotoxin in vitro

Monosaccharide[a]	Concentration (mM)	Cytopathic effect[b]
N-Acetyl-D-glucosamine	400	lysis
N-Acetyl-D-galactosamine	125	complete protection
	25	partial protection
N-Acetylneuraminic acid	125	complete protection
	25	partial protection
D-Galactose	250	lysis
L-Fucose	250	lysis
Muramic acid	75	lysis
Galactosamine	250	lysis
D-Glucose	250	lysis
D-Mannose	250	lysis

[a] Monosaccharides and activated soluble δ-endotoxin were incubated together for 60 min at 20°C before addition to cells

[b] Cytopathology observed in CF1 cells at a δ-endotoxin concentration of 50 $\mu g \cdot ml^{-1}$

10 $nmol \cdot ml^{-1}$ packed cells, indicating that NeuNAc is absent from this cell line.

Many toxins and other biological ligands are known to bind to specific glycoprotein or glycolipid receptors in the plasma membrane. The results reported above lead to the hypothesis that binding of the lepidopteran-specific toxin to a specific glycoconjugate on the plasma membrane of susceptible cells is an essential feature of its cytolytic action. Since GalNAc completely neutralised the toxin it seems likely to be part of the receptor. WGA and SBA both bind terminal GalNAc, therefore their observed ability to protect CF1 cells from the toxin may be explained by competition between lectin and toxin for the same binding site on the cell surface. Other toxins known to bind specific glycoconjugate receptors, such as shigella toxin [24], ricin [25] and cholera

Table 4

Effect of preincubation with lectins on toxicity of soluble δ-endotoxin in vitro

Lectin[a]	Sugar binding specificity	Cytopathic effect[b]
Concanavalin A	α-D-mannose > α-D-glucose	lysis
Peanut agglutinin	β-D-galactose	lysis
Soybean agglutinin	*N*-acetylgalactosamine	protection
Wheat germ agglutinin	*N*-acetylglucosamine > *N*-acetylneuraminic acid > *N*-acetylgalactosamine	protection
Ulex europaeus agglutinin	α-L-fucose	lysis

[a] Lectins and activated soluble δ-endotoxin were incubated together for 60 min at 20°C before addition to cells

[b] Cytopathology observed in CF1 cells at a δ-endotoxin concentration of 50 $\mu g \cdot ml^{-1}$

toxin [26] can also be inhibited by specific lectins or sugars.

Those toxins which must cross the plasma membrane generally show a time lag before the first symptoms of toxicity are observed. For example, although in a cell-free system diphtheria toxin will inhibit protein synthesis immediately, in intact cells a time lag of at least 40 min occurs before protein synthesis is inhibited [27]. In contrast, the first effects of *B. thuringiensis* var. *kurstaki* δ-endotoxin occur within 1 min [6,10].

The δ-endotoxin of *B. thuringiensis* var. *israelensis* binds specific phospholipids in the plasma membrane and causes rapid cytolysis by what has been suggested to be a detergent-like rearrangement of these lipids [13]. The precise mechanism of action of the lepidopteran-specific toxin of *B. thuringiensis* var. *kurstaki* has yet to be elucidated, but it appears that after binding to a plasma membrane receptor the membrane is rapidly made leaky to small ions and larger molecules. Possible mechanisms of action for the δ-endotoxin have recently been reviewed [28].

Further experiments are now in progress to isolate and characterise the GalNAc-containing receptor for the lepidopteran-specific δ-endotoxin of *Bacillus thuringiensis* var. *kurstaki*.

ACKNOWLEDGEMENTS

This work was supported by the Science and Engineering Research Council, and by the Agricultural Research Council. We thank Lesley Futter for editorial assistance.

REFERENCES

[1] Lecadet, M. and Martouret, D. (1962) C.R. Acad. Sci. 254, 2457–2459.
[2] Faust, R.M., Hallam, G.M. and Travers, R.S. (1974) J. Invertebr. Pathol. 24, 365–373.
[3] Heimpel, A.M. and Angus, T.A. (1960) Bacteriol. Rev. 24, 266–288.
[4] Ebersold, H.-R., Lüthy, P., Geiser, P. and Ettlinger, L. (1978) Experientia 34, 1672.
[5] Endo, Y. and Nishiitsutsuji-Uwo, J. (1980) J. Invertebr. Pathol. 36, 90–103.
[6] Percy, J. and Fast, P.G. (1983) J. Invertebr. Pathol. 41, 86–98.
[7] Murphy, D.W., Sohi, S.S. and Fast, P.G. (1976) Science 194, 954–956.
[8] Nishiitsutsuji-Uwo, J., Endo, Y. and Himeno, M. (1979) J. Invertebr. Pathol. 34, 267–275.
[9] Ebersold, H.-R., Lüthy, P. and Huber, H.E. (1980) Experientia 36, 495–496.
[10] Fast, P.G. and Donaghue, T.P. (1971) J. Invertebr. Pathol. 18, 135–138.
[11] Yamamoto, T. and McLaughlin, R.E. (1981) Biochem. Biophys. Res. Commun. 103, 414–421.
[12] Thomas, W.E. and Ellar, D.J. (1983) J. Cell Sci. 60, 181–197.
[13] Thomas, W.E. and Ellar, D.J. (1983) FEBS Lett. 154, 362–368.
[14] Stewart, G.S.A.B., Johnson, K., Hagelberg, E. and Ellar, D.J. (1981) Biochem. J. 196, 101–106.
[15] Folch, J., Lees, M. and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497–509.
[16] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[17] Warren, L. (1959) J. Biol. Chem. 234, 1971–1975.
[18] Bulla, L.A., Kramer, K.J. and Davidson, L.I. (1977) J. Bacteriol. 130, 375–383.
[19] Chestukhina, G.C., Zalunin, I.A., Kostina, L.I., Kotova, T.S., Katrukha, S.P., Lyublinskaya, L.A. and Stepanov, V.M. (1978) Biokhimiya 43, 681–686.
[20] Tojo, A. and Aizawa, K. (1983) Appl. Environ. Microbiol. 45, 576–580.
[21] Warren, L. (1963) Comp. Biochem. Physiol. 10, 153–171.
[22] Stollar, V., Stollar, B.D., Koo, R., Harrap, K.A. and Schlesinger, R.W. (1976) Virology 69, 104–115.
[23] Butters, T.D. and Hughes, R.C. (1981) Biochim. Biophys. Acta 640, 655–671.
[24] Keusch, G.T. and Jacewicz, M. (1977) J. Exp. Med. 146, 535–546.
[25] Olsnes, S., Refsnes, K. and Pihl, A. (1974) Nature 249, 627–631.
[26] Cuatrecasas, P. (1973) Biochemistry 12, 3547–3557.
[27] Uchida, T., Pappenheimer, A.M. and Harper, A.A. (1973) J. Biol. Chem. 248, 3845–3850.
[28] Lüthy, P. and Ebersold, H.-R. (1981) in: Pathogenesis of Invertebrate Microbial Diseases (Davidson, E.W. ed.) pp.235–267, Allanheld, Osmun and Co., USA.