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Interaction between the δ-endotoxin produced by *Bacillus thuringiensis* ssp. *entomocidus* and liposomes

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The δ-endotoxin produced by *Bacillus thuringiensis* ssp. *entomocidus* induced the release of encapsulated [^{14}C]sucrose from reverse-phase vesicles composed of phosphatidylcholine and cholesterol. No such release was detected when the phospholipid component of the vesicles was either phosphatidylethanolamine, phosphatidylglycerol, or sphingomyelin. The toxin-induced release was competitively inhibited by negatively charged organic ions while positively charged organic ions, apart from choline chloride, had no such effect. The existence of a polar head group in the phospholipid as well as intermolecular hydrogen bonding at the membrane surface, was found to be of major importance in the toxin-liposome interaction.

δ-Endotoxin; Sucrose release; Reverse-phase vesicle; Charged-group inhibition; Toxin-receptor interaction; (*Bacillus thuringiensis* ssp. *entomocidus*, *Spodoptera littoralis*)

1. INTRODUCTION

The crystalline proteinaceous δ-endotoxin produced by various strains of *Bacillus thuringiensis* during sporulation is responsible for the insecticidal properties of this bacterium [1–4]. It is generally agreed that the δ-endotoxin acts primarily on the midgut epithelial cell membrane of the susceptible Lepidopteran and Dipteran larvae, inducing drastic histological changes which result in swelling and lysis [5–15].

Employing insect cell culture techniques, Thomas and Ellar [21] had shown that the Dipteran toxin produced by *B. thuringiensis* ssp. *israelensis* lost its cytolytic activity upon binding to multilamellar liposomes composed of phospholipid with a zwitterionic polar head group. In contrast, the Lepidopteran toxin produced by *B. thuringiensis* ssp. *kurstaki* had shown no binding affinity to vesicles, but was inactivated by preincubation with *N*-acetylgalactosamine or *N*-acetylneuraminic acid [22]. Furthermore, the recent discovery of a glycoprotein containing *N*-acetylgalactosamine in plasma membranes of *Choristoneura fumiterana* cells that bind the *B. thuringiensis* ssp. *kurstaki* toxin [23] suggests a relatively specific binding mechanism of this toxin with a membrane receptor [22,23]. The Lepidopteran δ-endotoxin produced by *B. thuringiensis* ssp. *entomocidus* is a 64 kDa protein which interacts with an octyl-Sepharose 4B gel in a specific, reproducible pattern. This protein tends to form high molecular mass aggregates in aqueous solution [12,24]. The toxin from *B. thuringiensis* ssp. *entomocidus* has a high cytolytic activity on larval midgut epithelial cells of *Spodoptera littoralis* [4,12,16]. It retains its activity at pH 7.5, this value being the actual value measured on the surface of the midgut epithelial cell membrane [25].

Here, we have investigated the effect of the *B. thuringiensis* ssp. *entomocidus* δ-endotoxin on the release of encapsulated [^{14}C]sucrose from

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reversed-phase vesicles composed of various phospholipids together with cholesterol and the modulation of that release by different competitive inhibitors.

2. MATERIALS AND METHODS

Phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, sphingomyelin, cholesterol, glycerol 3-phosphate, choline chloride, Triton N-101, sodium cholate and sodium lauryl sulphate were obtained from Sigma (St. Louis, MO). Amino acids were purchased from Merck (Darmstadt). [^{14}C]Sucrose (spec. act. 0.56 Ci/mmol) was obtained from Amersham (Bucks, England).

2.1. *Preparation of the δ-endotoxin*

The toxic fraction of 64 kDa was purified from *B. thuringiensis* ssp. *entomocidus* endotoxin crystals as described [12,24]. Residues of detergents were removed from the purified toxin by gel filtration on a Sepharose 6B column previously equilibrated with 10 mM Tris-HCl, pH 8.5. The purified fraction was highly insecticidal against 2nd instar larvae of *S. littoralis*, as determined by the bioassay procedure in [12].

2.2. *Preparation of reverse-phase evaporation vesicles (REV)*

Large unilamellar vesicles were prepared according to the REV technique [26]. Aliquots of 88 μmol phospholipid and cholesterol at a 1:1 molar ratio dissolved in 5 ml chloroform were evaporated in a rotary evaporator to form a thin-layer film and then flushed with N_2. The lipids were redissolved in 3 ml diethyl ether and 1 ml of 0.8 mM phosphate buffer (PB, pH 7.5) was added. The aqueous phase contained 4–10 μCi [^{14}C]sucrose depending on the encapsulation cavity of the vesicles with respect to the phospholipid component. The two-phase system was sonicated for about 10 s using a microtip sonicator, until the mixture had become a one-phase dispersion. The organic solvent was removed by rotary evaporation. The unincorporated lipids and unencapsulated sucrose were removed by 15 min centrifugation at 23000 × *g*. The pellet was washed twice with 0.8 mM PB (pH 7.5) and resuspended in 10 ml of the same buffer.

The total radioactivity encapsulated in a given volume of liposomes whether composed of phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine or sphingomyelin was essentially equal.

2.3. *Measurement of [^{14}C]sucrose release from toxin-treated REV*

Aliquots of 0.2 ml liposome suspension were mixed with 0.1 ml of 0.8 mM PB (pH 7.5) containing the toxin at increasing concentrations (0.1–10.0 μM). After 15 min incubation at 37°C, the suspension was centrifuged at 12000 × *g* for 4 min. Aliquots of 0.1 ml supernatant were added to 4 ml scintillation cocktail for radioactivity counting. Liposomes incubated with inactivated toxin served as a control. Inactivation was performed by pretreatment for 2 h at pH 2.0.

2.4. *Inhibition experiments*

Aliquots of glycerol 3-phosphate, choline chloride and various amino acids were added to 0.2 ml liposome suspension at final concentrations ranging between 10 and 200 μM. Then toxin was added at a final concentration of 2.5 μM. The final volume of the reaction mixture, incubation time and temperature were as described above.

Protein was determined using the method of Bradford [27] with bovine serum albumin as standard.

3. RESULTS AND DISCUSSION

As shown in fig.1, the δ-endotoxin produced by *B. thuringiensis* ssp. *entomocidus* induced the release of [^{14}C]sucrose encapsulated in REV composed of phosphatidylcholine and cholesterol. The radioactivity released after incubation with 10 μM toxin was 80% of the total radioactivity encapsulated. Nonionic detergents (mixture of 0.1% sodium cholate and 0.1% Triton N-101) as well as anionic detergent (1% SDS) released practically 100% of the marker encapsulated in these vesicles.

Interaction of the toxin with liposomes composed of phosphatidylglycerol and cholesterol resulted in the release of only 20% of the total radioactivity released by detergents, whether anionic or nonionic. Phosphatidylethanolamine and sphingomyelin liposomes showed resistance to the toxin activity (fig.1), as well as to the influence of nonionic detergents. However, 1% SDS induced 100% release from phosphatidylethanolamine liposomes while only 40% was recorded for sphingomyelin vesicles. The value of a maximal release of 20% from phosphatidylglycerol vesicles was not statistically different ($P < 0.05$) from that recorded for phosphatidylethanolamine and sphingomyelin liposomes.

The results thus far indicate that electrostatic forces are involved in the interaction between toxin and liposomes. Thus, the essential structural characteristic that is required of the phospholipid component for interaction with the toxin is a positive charge on the polar head group. Apart from phosphatidylcholine, both sphingomyelin and phosphatidylethanolamine meet this requirement. The failure of the toxin to induce leakage of radioactive marker from the sphingomyelin liposomes may, very likely, be due to the stability of the sphingomyelin molecule. The presence of hydroxyl groups, amide bonds and a *trans* double bond in the interfacial region of sphingomyelin provides a series of hydrogen bonds within the lipid bilayer that results in increased stability and

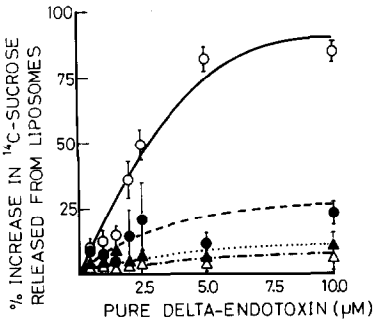

Fig.1. Release of encapsulated [^{14}C]sucrose from reverse-phase vesicles composed of phospholipid and cholesterol in a 1:1 molar ratio, induced by 15 min incubation at 37°C with the δ-endotoxin produced by *B. thuringiensis* ssp. *entomocidus*. Phospholipids: phosphatidylcholine (○), phosphatidylglycerol (●), phosphatidylethanolamine (▲), sphingomyelin (△). Results are means ± SD of 4 determinations. Bars correspond to 1 SD.

reduced permeability of the membrane [28]. The lack of release of internal marker in phosphatidylethanolamine and cholesterol vesicles might also be due to the high stability that results from intermolecular hydrogen bonding at the membrane surface [29].

Table 1

The effect of various organic ions on the release of encapsulated [^{14}C]sucrose from phosphatidylcholine/cholesterol liposomes after incubation with pure δ-endotoxin

Organic ion	cpm per 100 μl of reaction mixture 12000 × *g* supernatant	Inhibition (%) of toxin-induced [^{14}C]sucrose release
Absent	2508 ± 118[a]	0
Glycerol phosphate	173 ± 100[b]	93
Glutamate	832 ± 212[c]	67
Cysteic acid	1209 ± 444[c]	52
Choline chloride	1207 ± 290[c]	52
Aspartate	1357 ± 379[c]	46
Lysine	2301 ± 506[a]	8
Leucine	2330 ± 776[a]	7
Phenylalanine	2722 ± 359[a]	−9
Arginine	2778 ± 590[a]	−11
Glycine	3118 ± 652[a]	−24

Results are means ± SD of 4 determinations. Means denoted by the same superscript are not significantly different ($P < 0.01$). Concentration of glycerol 3-phosphate and choline chloride was 100 μM while that of all other ions was 200 μM; toxin concentration was 2.5 μM; toxin/lipid molar ratio was 1:1400; incubation was carried out for 15 min at 37°C in 0.8 mM phosphate buffer, pH 7.5

The involvement of electrostatic forces in the toxin-liposome interaction is supported by the results summarized in table 1. Negatively charged organic ions inhibited toxin activity, while of the positively charged ions, only choline chloride, the positively charged and methylated component of the phospholipid head group, had a distinguishable inhibitory effect. The inhibition of toxin activity by negatively charged organic ions may, very likely, be the result of competition on the quaternary ammonium of the membrane phospholipid component. On the other hand, the finding that of the positively charged organic ions only choline chloride had a significant inhibitory effect may be the result of a specific affinity for choline in the endotoxin sequence. This sort of electrostatic toxin-membrane interaction should be reversible and was indeed found to be so as shown in the data presented in fig.2.

There was no need for incorporation into the liposomes of any carbohydrate moiety that would serve as an acceptor in order to allow toxin-induced [^{14}C]sucrose release. In this respect, *B. thuringiensis* ssp. *entomocidus* δ-endotoxin does not resemble cholera toxin which induced marker release from liposomes only when they contained the glycolipid Gm_1 [30,31].

The mode of action thus proposed is therefore

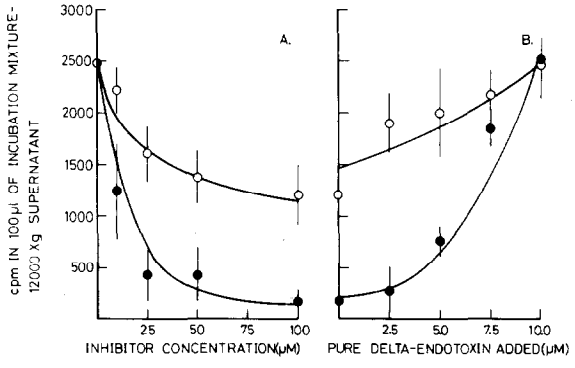

Fig.2. (A) Inhibition of toxin-induced release of [^{14}C]sucrose from phosphatidylcholine and cholesterol liposomes in a 1:1 molar ratio by chloride salts of glycerol 3-phosphate (●) and choline (○). The concentration of the δ-endotoxin produced by *B. thuringiensis* ssp. *entomocidus* was 2.5 μM. (B) Reversal of the inhibitory effect produced by chloride salts of glycerol 3-phosphate (●) and choline (○) at a final concentration of 25 μM by increasing the concentration of δ-endotoxin. Results are means ± SD of 3 determinations. Bars correspond to 1 SD.

nonspecific, although Lepidopteran and Dipteran larvae differ in the phosphatidylcholine/phosphatidylethanolamine ratio characteristic of their membranes [32]. However, carbohydrate moieties can be involved in the primary binding process and may serve as determinants of the species-specific phase. Furthermore, binding and activity are not always linked as exemplified by the *B. thuringiensis* ssp. *thuringiensis* δ-endotoxin which binds to *Drosophila melanogaster* cell lines without disturbing viability [33].

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