Lipid-induced Polymerization of Staphylococcal α -Toxin

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SUMMARY

The ability of certain lipids to induce polymerization of staphylococcal α -toxin has been investigated using polyacrylamide disc gel electrophoresis in the presence of sodium dodecyl sulphate to monitor conversion of the monomeric form of α -toxin (molecular weight = 36 000) to the α_{128} aggregate. The widely differing lipids varied in the order diglyceride > lecithin > cholesterol > lysolecithin in polymer-inducing activity. No individual component was as efficient as a mixed dispersion of lecithin, cholesterol and dicetyl phosphate (molar ratio 70:10:20) in inducing polymerization. In general our observations indicate that there is no specific lipid inducer and that the outcome of the interaction between staphylococcal α -toxin and biological membranes probably depends on the location of lipids in the membrane and their distribution in relation to one another. Also the results with diglyceride confirm our earlier suggestion that α -toxin can react hydrophobically with lipids.

INTRODUCTION

The α -toxin of *Staphylococcus aureus* occurs in several different molecular forms. Purified toxin preparations, analysed by ultracentrifugation, contain approximately 90 % of a slow moving component (α_{38}) and 10 % of an aggregated form (α_{128}) (Arbuthnott, Freer & Bernheimer, 1967). The molecular weight of the α_{38} component, which is the biologically active form of the toxin, has been estimated as 33 000 to 36 000 (Forlani, Bernheimer & Chiancone, 1971; McNiven, Owen & Arbuthnott, 1972).

Previous studies (Weissmann, Sessa & Bernheimer, 1966; Freer, Arbuthnott & Bernheimer, 1968) on the activity of this cytolytic toxin against artificial lipid membranes (liposomes), consisting of lecithin, cholesterol and charged fatty acid derivatives, showed the following: (i) disruption of the lamellar structure and release of sequestered marker molecules; (ii) appearance on membrane surfaces of ring structures morphologically identical with α_{128} ; and (iii) disappearance of toxic activity. Similar effects have been observed following the interaction of staphylococcal α -toxin with mammalian erythrocytes (Freer *et al.* 1968). Other cytolytic toxins known to react with liposomes or dispersed lipids include staphylococcal δ -haemolysin (Kreger, Kim, Zaboretsky & Bernheimer, 1971), staphylococcal leucocidin (Woodin, 1970), streptolysin S (Bangham, Standish & Weissmann, 1965), streptolysin O (Howard, Wallace & Payling Wright, 1953), and a lytic polypeptide from bee venom, melittin (Sessa, Freer, Colacicco & Weissmann, 1969). Indeed, there is increasing indirect evidence that the lytic action of staphylococcal α -toxin (Buckelew & Colacicco, 1971; Colacicco & Buckelew, 1971) and possibly other cytolytic toxins (Oberley & Duncan, 1971) can be accounted for in part by the ability of these agents to penetrate lipid components of cell membranes.

The factors which govern the binding and polymerization of staphylococcal α -toxin following its interaction with erythrocyte membranes and liposomes are not understood.

The influence of individual lipid components on polymerization was the object of this investigation. Information gained from this approach may contribute towards an understanding of the mechanism of action of this toxin.

Polymerization to the α_{128} ring form was monitored by electron microscopy and polyacrylamide disc gel electrophoresis in the presence of sodium dodecyl sulphate (SDS). It has been reported that this agent solubilizes biological membranes forming lipid/detergent and protein/detergent complexes (Engelman, Terry & Morowitz, 1967; Salton, 1968; Fox & Keith, 1972). The method of SDS polyacrylamide disc gel electrophoresis has been used to separate membrane polypeptides according to molecular weight in samples of SDS solubilized membranes (Berg, 1969; Bender, Garan & Berg, 1971; Bretscher, 1971*a, b*; Wallach, 1972). This technique is suitable for the present study because: (i) it allows dissociation of protein/lipid complexes; (ii) it gives an approximate value for the molecular weight of proteins (Maurer, 1971); and (iii) it allows resolution of the α_{128} polymer which resists dissociation by SDS (Forlani *et al.* 1971).

METHODS

Staphylococcal α -toxin. Toxin produced by *Staphylococcus aureus* strain Wood 46 (NCTC 7121) was purified by the method of McNiven *et al.* (1972). This entailed isoelectric focusing of crude ammonium sulphate precipitated toxin in a broad pH gradient (pH 3 to 10) stabilized in a density gradient of sucrose, using the LKB 8102 (440 ml) column. The main α -toxin component (α_a) so obtained was then refocused under the same conditions using the LKB 8101 (110 ml) column. The peak fraction of α_a (pI = 8.55) obtained after refocusing was used throughout. No contaminating staphylococcal products (lipase (tributyrylase), egg-yolk factor, fibrinolysin, coagulase, phosphatase, gelatinase, β -toxin and δ -toxin) could be detected in this fraction and in double diffusion tests against commercial α -antitoxin (Wellcome Research Laboratories, Beckenham, Kent) a single precipitin line was formed. By SDS polyacrylamide disc gel electrophoresis the preparation contained a major component with a mol. wt of 36000 and a trace of material having an approximate mol. wt of 170000 (McNiven *et al.* 1972). Rabbit erythrocytes were used to assay haemolytic activity (McNiven *et al.* 1972).

Lipid components. Ovocleithin was purified from fresh hens' eggs by the method of Papahadjopoulos & Miller (1967). Lyophilized egg yolk was extracted three times with acetone at 0 °C. The acetone insoluble residue was extracted three times with chloroform + methanol (1:1); these extracts were pooled and dried *in vacuo*. This dried material was dissolved in petroleum ether (boiling range = 60 to 80 °C) and precipitated with acetone at 0 °C. Acetone insoluble material was collected by centrifugation, redissolved in petroleum ether and allowed to stand for 16 h at 0 °C. The precipitate which formed in the cold was discarded; the supernatant fluid was dried *in vacuo* and stored under nitrogen. This crude lecithin preparation was purified by column chromatography first on alumina and then on silicic acid as follows. A solution of 200 mg of crude egg phospholipid in 10 ml chloroform + methanol (1:1) was applied to a 10 mm diam. column containing 10 g of activated alumina and was eluted at a flow rate of approximately 10 ml/min. Fractions of 0.5 ml were collected and monitored by thin-layer chromatography (t.l.c.) on silicic acid G (250 micron thickness) using chloroform + methanol + water (65:25:4) as solvent. T.l.c. plates were developed by spraying with 50 % (v/v) sulphuric acid followed by heating at 110 °C for 30 min. Fractions containing lecithin were pooled and dried *in vacuo* and stored under nitrogen. This material, dissolved in chloroform + methanol (70:30) was applied to activated silicic acid (24 g)

packed in a 10 mm diam. column and was eluted with the same solvent. Fractions of 2 ml were collected and monitored by t.l.c. as described above. Those containing only lecithin were pooled, dried *in vacuo* and stored as a solution in chloroform at -20°C .

Diglyceride was prepared from lecithin in the following manner. To 0.4 ml of an aqueous dispersion of ovolecithin (3.8 mg) were added 0.02 ml (33 μg) of highly purified *Clostridium perfringens* phospholipase C (a gift from Mr C. J. Smyth of this department) and 0.05 ml of 0.05 M-calcium acetate. After 2 h of incubation at 37°C , the diglyceride, which floated as a layer, was removed and washed several times with distilled water. It was dissolved in 0.1 ml acetone and used immediately; by t.l.c. the diglyceride preparation was free of detectable lecithin.

Other lipids included crystalline, chromatographically pure egg yolk lysolecithin (Koch-Light, Colnbrook, Buckinghamshire), dicetyl phosphate (K & K Laboratories, Plainview, New York, U.S.A.) and cholesterol (B.D.H., Poole, Dorset).

Lipid dispersions. Liposomes composed of lecithin, cholesterol and dicetyl phosphate in the molar proportions 70:10:20 were prepared as described previously (Freer *et al.* 1968) to give a total lipid concentration of 12.1 mg/ml. Dispersions of lecithin and lysolecithin as single components contained 10 mg/ml; both were dispersed in distilled water by sonicating for 90 s in an ultrasonic bath (Sonicor Instrument Corporation, Glen Head, New York, U.S.A.). Diglyceride and cholesterol were dispersed by adding acetone solutions of the appropriate lipid to distilled water heated to 95°C . The concentration of diglyceride and cholesterol was adjusted to correspond approximately to the molar proportions of lecithin and cholesterol present in liposomes.

Reaction mixtures. With the exception of phospholipase experiments, reaction mixtures contained 0.2 ml lipid dispersion, 0.1 ml 0.03 M-sodium borate, pH 8.3, and 0.1 ml staphylococcal α -toxin [100 μg protein; 2500 Haemolytic Units (HU)]. After 30 min incubation at 37°C samples were prepared for polyacrylamide disc gel electrophoresis.

Phospholipase C experiments. To 0.2 ml of liposomes were added 0.01 ml 0.1 M-calcium acetate and 33 μg purified phospholipase C in 0.02 ml distilled water (1 mg phospholipase C released 4 μmol phosphate/min from lecithin at 37°C). After incubating at 37°C for 30 min, 100 μg of purified staphylococcal α -toxin was added as described above; the mixture was incubated for a further 30 min at 37°C . Appropriate control mixtures were also prepared.

SDS polyacrylamide disc gel electrophoresis. Reaction mixtures were diluted to 1 ml with 0.03 M-sodium borate buffer, pH 8.3, and were made 1.5 % (w/v) with SDS, 1.2 % (v/v) with mercaptoethanol and 0.1 ml glycerol was added to give a final volume of 1.3 ml; 0.1 ml of sample was then layered on each gel. Electrophoresis was performed by the method of McNiven *et al.* (1972) using a separating gel containing 11.7 % (w/v) acrylamide (B.D.H.) and 0.153 % (w/v) *NN'*-methylenebisacrylamide (B.D.H.); $T = 11.8\%$, $C = 1.3\%$ (Hjerten, 1962). Stacking gels were strengthened by a modification of the method of DeVito & Santomé (1966) omitting ethylene diamine tetraacetate. A current of 1 mA/gel was applied and when the tracking dye (bromophenol blue) reached 10 mm from the end of the gel the current was increased to 5 mA/gel for 15 min; the total running time was 2.75 ± 0.1 h. Gels were fixed, stained and destained as described previously (McNiven *et al.* 1972). Each sample was run in quadruplicate and a mixture of proteins of known mol. wt was included in each run (McNiven *et al.* 1972). Densitometer traces of stained gels were obtained using a Joyce-Loebl u.v. Polyfrac scanner at 265 nm linked to a Bryans potentiometric recorder.

Electron microscopy. Samples of reaction mixtures were examined after negative staining with 2 % (w/v) ammonium molybdate in distilled water in a Phillips EM 300 electron microscope operating at 60 kV.

Fig. 1. Densitometer traces of SDS polyacrylamide disc gels of reaction mixtures (Methods) containing: (a) staphylococcal α -toxin alone; (b) liposomes (lecithin + cholesterol + dicetyl phosphate, 70:10:20); and (c) α -toxin + liposomes. Traces were recorded on a Bryans recorder set at 4V at a speed of 1 mm/s using a Joyce-Loebl u.v. Polyfrac scanner. A, fast moving α -toxin component (mol. wt = 36000); B, slow moving α -toxin component (mol. wt = approximately 170000); C, diffuse area of absorption due to liposomes. $\uparrow$, Position of the tracking dye bromophenol blue; $\uparrow$, gel artefact seen in all SDS disc gels; +, anode; -, cathode.

RESULTS

SDS disc gel electrophoresis of purified staphylococcal α -toxin (Fig. 1a) contained a major fast moving component (peak A) and a trace of a slow moving component (peak B); these had mol. wts of 36000 and approximately 170000 respectively as described previously (McNiven *et al.* 1972). After interaction with liposomes (Fig. 1c) there was a pronounced increase in peak B and a corresponding decrease in peak A. When unstained gels were sliced at 1 mm intervals and extracted with a small volume of distilled water many ring structures identical to α_{128} toxin (Arbuthnott *et al.* 1967) were detected in slices which corresponded to the position of peak B. Moreover, negative staining of toxin-treated liposomes showed fragments with numerous attached ring structures, identical to those reported in our earlier publication (Freer *et al.* 1968). These findings indicate that the slow moving component in SDS disc gels corresponds to α_{128} .

In electrophoresis of liposomes alone an unstained zone of opacity (peak C) which overlapped with the artefact band appeared after fixation of gels (Fig. 1b); in disc gels of toxin/liposome mixtures the position of peak C was unchanged (Fig. 1c). The reason for its appearance is not known. However it was absent in samples containing phospholipase C-treated liposomes and in those of individual lipids.

In order to assess the role of lecithin in the polymerization reaction, preliminary experiments

Fig. 2. Densitometer traces of SDS polyacrylamide disc gels of reaction mixtures (Methods) containing: (a) phospholipase C alone; (b) staphylococcal α -toxin alone; and (c) phospholipase C-treated liposomes + α -toxin. Traces were recorded as for Fig. 1. A, fast moving α -toxin component; B, slow moving α -toxin component. $\uparrow$, Position of tracking dye, bromophenol blue; $\uparrow$, gel artefact; +, anode; -, cathode.

Table 1. Influence of different lipids on the polymerization of staphylococcal α -toxin

Experiment	Reaction mixture	Polymer present* (%)	$\frac{\% \text{ Polymer in } T_L \dagger}{\% \text{ Polymer in } T_a}$ (%)
1	α -toxin	5.3	—
	α -toxin + cholesterol	15.2	2.9
	α -toxin + diglyceride	38.6	7.3
	α -toxin + liposomes	48.3	9.2
2	α -toxin	7.1	—
	α -toxin + liposomes + Ca^{2+} + phospholipase C	61.4	8.6
3	α -toxin	4.2	—
	α -toxin + lecithin	12.8	3.1
4	α -toxin	7.6	—
	α -toxin + lysolecithin	14.3	1.9

* Percentage values were calculated from areas of peaks A and B on densitometer traces of disc electrophoresis gels.

$\dagger T_a = \alpha$ -toxin alone; $T_L = \text{toxin} + \text{lipid}$.

Fig. 3. Electron micrographs of negatively stained liposomes: (a) incubated for 30 min at 37 °C in the presence of 0.005 M-calcium acetate; (b) incubated for 30 min at 37 °C in the presence of 0.005 M-calcium acetate and phospholipase C; (c) liposomes treated as in (b) but followed by incubation with staphylococcal α -toxin (Methods). Arrows indicate α_{12S} polymer. Bar represents 100 nm.

Fig. 4. Densitometer traces of SDS polyacrylamide disc gels of reaction mixtures (Methods) containing: (a) α -toxin alone; (b) diglyceride derived from ovoidlecithin; and (c) α -toxin + diglyceride. Traces were recorded as for Fig. 1. A, fast moving α -toxin component; B, slow moving α -toxin component. $\uparrow$, Position of tracking dye, bromophenol blue; $\uparrow$, gel artefact; +, anode; - cathode.

were carried out to determine whether treatment of liposomes with highly purified phospholipase C, prior to the addition of α -toxin, affected the formation of α_{128} . From Fig. 2 and Table 1 it can be seen that such treatment did not significantly affect the ability of the lipid mixture to induce polymer formation as assessed by SDS disc gel electrophoresis. However electron microscopy of phospholipase C-treated liposomes after addition of α -toxin showed that polymerization occurred preferentially on certain lipid globules (Fig. 3c). Such areas, densely packed with polymerized toxin, contrasted with the dispersed distribution of polymer reported previously for α -toxin-treated liposomes (Freer *et al.* 1968). In the absence of α -toxin, liposomes and phospholipase C-treated liposomes could not be differentiated by their morphologies (Fig. 3a, b).

The results of experiments designed to investigate the ability of individual lipids to induce polymerization are summarized in Table 1 and Fig. 4. The relative changes in peaks A and B were determined by measuring the peak areas of the α -toxin components in densitometer traces of stained electrophoresis gels. The actual traces for the experiment utilizing diglyceride are shown in Fig. 4. In all experiments peaks A and B were clearly resolved and traces were of comparable quality to those shown in Fig. 4. Peaks A and B were cut from traces and weighed in order to estimate the relative amounts of each component present. The amount of peak B is expressed as a percentage of the total toxin present in each gel. This method, although not strictly quantitative, proved useful in comparing individual lipid components (Table 1); values for liposomes and phospholipase C-treated liposomes are included for comparison. Lecithin, lysolecithin and cholesterol induced a two- to threefold increase in peak B whereas diglyceride induced approximately a sevenfold increase. This compared

Fig. 5. Electron micrograph of a negatively stained cholesterol dispersion after incubation (a) in the absence of and (b) in the presence of staphylococcal α -toxin (Methods). Arrows indicate α_{128} polymer. Bar represents 100 nm.

Fig. 6. Electron micrograph of a negatively stained lecithin dispersion after incubation (a) in the absence of and (b) in the presence of staphylococcal α -toxin (Methods). Arrows indicate α_{128} polymer. Bar represents 100 nm.

Fig. 7. Electron micrographs of a negatively stained dispersion of diglyceride after incubation (a) in the absence of and (b) in the presence of staphylococcal α -toxin (Methods). Arrows indicate α_{128} polymer. Bar represents 100 nm.

with an eight- to ninefold increase for liposomes and phospholipase C-treated liposomes. Electron micrographs of various toxin lipid mixtures are shown in Fig. 5, 6 and 7. Cholesterol and diglyceride dispersions contained spherical globules having no characteristic substructure, whereas lecithin showed a lamellar appearance typical of aqueous phospholipid dispersions. After treatment with staphylococcal α -toxin, α_{128} rings were observed in every case and appeared most numerous in α -toxin/diglyceride mixtures. These findings are in agreement with the results obtained using disc gel electrophoresis.

DISCUSSION

Our earlier suggestion, that the interaction of staphylococcal α -toxin with liposomes leads to conversion of α_{38} toxin to the α_{128} polymer, is supported by the results of SDS polyacrylamide disc gel electrophoresis. The high molecular weight component, peak B, consists of ring-like aggregates similar to α_{128} toxin and appeared in increased amounts on interaction of staphylococcal α -toxin with liposomes and individual lipids.

The lipids tested were of widely differing chemical structure and each was found capable of inducing polymerization of the toxin as assessed by monitoring the increase in peak B. However, individual lipids differed considerably in the efficiency with which they induced polymer formation (diglyceride > lecithin > cholesterol > lysolecithin). The appearance of densely packed toxin polymer on certain globules in phospholipase C-treated liposomes, after addition of α -toxin (Fig. 3c), resembled that observed in mixtures of α -toxin and diglyceride (Fig. 7b). It seems likely that the polymer-covered globules shown in Fig. 3(c) correspond to areas rich in diglyceride. Although diglyceride caused a sevenfold increase in

peak B, no single lipid component was as efficient as either the complete liposome mixture or phospholipase C-treated liposomes in causing polymerization. Therefore polymer formation cannot be explained in terms of a single lipid inducer. It seems likely that the location and orientation of lipid components in relation to one another in artificial and natural membranes determines the degree of polymerization of staphylococcal α -toxin.

Of the individual lipids tested, it is notable that diglyceride was the most active in inducing polymerization. This finding shows that the polar group of lecithin is not required for polymerization and adds support to the concept that staphylococcal α -toxin is capable of hydrophobic interaction with lipids (Freer *et al.* 1968). It must be emphasized however that the relationship between the formation of α_{128} by polymerization and the surface activity of α -toxin, as manifested by its penetration of air/water and lipid/water interfaces and biological membranes, remains to be clarified. In this respect it would be interesting to investigate toxin/lipid interactions in greater detail and in particular to determine whether polymer induction by diglyceride depends on the carbon chain length and degree of saturation of the fatty acid substituents.

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