Biochimica et Biophysica Acta, 648 (1981) 6–12
Elsevier/North-Holland Biomedical Press

BBA 79395

EFFECT OF STREPTOLYSIN S ON LIPOSOMES

INFLUENCE OF MEMBRANE LIPID COMPOSITION ON TOXIN ACTION

JAMES L. DUNCAN and LELA BUCKINGHAM

Department of Microbiology-Immunology, Northwestern University Medical and Dental Schools, Chicago, IL 60611 (U.S.A.)

(Received March 9th, 1981)

Key words: Streptolysin S; Liposome; Lipid composition; Toxin action

The effect of the bacterial cytolytic toxin, streptolysin S, on liposomes composed of various phospholipids was investigated. Large unilamellar vesicles containing [^{14}C]sucrose were prepared by reverse-phase evaporation, and membrane damage produced by the toxin was measured by following the release of labeled marker. The net charge of the liposomes had little or no effect on their susceptibility to steptolysin S and the toxin was about equally effective on liposomes composed of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidylglycerol. Experiments with liposomes composed of synthetic phospholipids showed that the ability of the toxin to produce membrane damage depended on the degree of unsaturation of the fatty acyl chains. The order of sensitivity was C18 : 2 phosphatidylcholine > C18 : 1 phosphatidylcholine > C18 : 0 phosphatidylcholine = C16 : 0 phosphatidylcholine. Liposomes containing the latter two phospholipids were virtually unaffected by streptolysin S, and experiments with C18 : 0 phosphatidylcholine suggested that toxin activity does not bind to liposomes composed of phospholipids with saturated fatty acyl chains. The inclusion of 40 mol% cholesterol in C16 : 0 phosphatidylcholine and C18 : 0 phosphatidylcholine liposomes made these vesicles sensitive to streptolysin S. Egg phosphatidylcholine liposomes, which were unaffected at 0°C and 4°C became susceptible to the toxin at these temperatures when cholesterol was included. Liposomes composed of C14 : 0 phosphatidylcholine were unaffected by streptolysin S at temperatures below the chain-melting transition temperature (23°C) of this phospholipid, but became increasingly susceptible above this temperature. The results suggest that the fluidity of the phospholipid hydrocarbon chains in the membrane is important in streptolysin S action.

Introduction

Streptolysin S is an unusual cytolytic toxin produced by *Streptococcus pyogenes* (reviewed in Refs. 1–3). The toxin, which can be found in a membrane-associated precursor form [4], is secreted as a peptide of 28–32 amino acids [5,6]. Toxic activity can be obtained in a soluble form only when the streptococcal cells are incubated in the presence of certain proteins, RNA, or nonionic detergents, agents which apparently act as carriers and/or stabilizers of the peptide. The toxin is also active in the presence of agar; the large clear zone of hemolysis that surrounds colonies of the organism growing on blood agar plates is due to streptolysin S.

In addition to erythrocytes, streptolysin S damages and lyses other eukaryotic cells as well as bacterial protoplasts. The toxin is thought to act directly on the cell membrane, but the nature of the lesion produced by streptolysin S is not completely understood. A number of phospholipids, including phos-

Abbreviations: C14 : 0, C16 : 0, C18 : 0, C18 : 1, C18 : 2 phosphatidylcholine represent dimyristoyl-, dipalmitoyl-, distearoyl-, dioleoyl- and dilinoleoylphosphatidylcholine; TEMPO, 2,2,6,6-tetramethylpiperidine-*N*-oxyl.

phatidylcholine and phosphatidylethanolamine, have been shown to inactivate streptolysin S [7], suggesting that phosphoglycerides are the target of toxin action. There is no evidence that the toxin has enzymatic activity, however [7,8]. The lysis of erythrocytes by streptolysin S occurs by a colloid osmotic process [9,10]. Small holes or channels are presumably produced by the toxin, allowing the free passage of ions across the membrane. The resulting net flow of ions and water into the cell results in osmotic lysis. Studies on the leakage of radioactive marker molecules from human diploid fibroblasts treated with streptolysis S has provided additional evidence that the toxin produces small, functional holes in the cell membrane [11].

Multilamellar phospholipid vesicles (liposomes) have been shown to be susceptible to streptolysin S [12]. Action of the toxin on phosphatidylcholine liposomes, which results in the release of entrapped ions or glucose was found to be unaffected by the presence of cholesterol in the vesicle membrane [13]. Recently, Szoka and Papahadjopoulos [14] described the preparation of large unilamellar and oligolamellar liposomes by a reverse-phase evaporation technique. The vesicles are able to encapsulate marker molecules with high efficiency, and their aqueous volume to lipid ratio is 4-times higher than that of multilamellar vesicles. This paper describes the effects of streptolysin S on large unilamellar liposomes prepared with phospholipids containing different polar head groups or hydrocarbon chains. The results indicate that the fluidity of the membrane is a determining factor in streptolysin S action.

Materials and Methods

Streptolysin S. Streptolysin S was prepared by incubating *S. pyogenes* strain S23g in Bernheimer's medium [15] containing yeast RNA core (Sigma), as previously described [9]. After chromatography on Sephadex G-50 and DEAE-Sephadex [9], the toxin was dialyzed in water, lyophilized and stored at −70°C. The specific activity of the toxin preparations used in these experiments was approx. $5 \cdot 10^5$ hemolytic units per mg protein.

For titration of streptolysin S activity, the toxin was diluted in phosphate-buffered saline, then incubated for 30 min at 37°C with an equal volume of a 1.7% rabbit erythrocyte suspension. Lysis was measured by determining hemoglobin release colorimetrically at 540 nm. One hemolytic unit of toxin is defined as the greatest dilution of streptolysin S that produces 50% hemolysis under these conditions.

Lipids. The following lipids were obtained from Sigma, analyzed by thin layer chromatography, and used without further purification: phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylserine, dicetyl phosphate, stearylamine, sphingomyelin, cholesterol, and the synthetic phospholipids C14 : 0 phosphatidylcholine, C16 : 0 phosphatidylcholine, C18 : 0 phosphatidylcholine and C18 : 1 phosphatidylcholine. C18 : 2 phosphatidylcholine was obtained from Applied Science Laboratories.

Liposome preparation. Large unilamellar vesicles were prepared by the reverse-phase evaporation method [14]. Appropriate lipid mixtures in chloroform or chloroform/methanol were evaporated under reduced pressure in a rotary evaporator. The lipids were redissolved in the organic phase by the addition of diethyl ether. Certain lipids with low solubility in ether required addition of chloroform and methanol. The aqueous phase consising of a low salt buffer (phosphate-buffered saline diluted 1 : 10) containing 25 μCi [^{14}C]sucrose (Schwarz/Mann) was added and the two-phase system was sonicated for 10 s using the microtip of a Branson sonifier. The resulting one-phase dispersion was returned to the rotary evaporator to remove the organic phase. The liposomes remained under vacuum for 2–16 h to ensure that the ether had been completely removed.

Liposomes were separated from unincorporated lipid and unentrapped sucrose on a 0.8 × 10 cm Sepharose 4B column equilibrated with low salt buffer at room temperature. The pH of the column buffer was raised to 8.0 for liposomes containing certain lipids to prevent them from sticking to the column. The liposomes eluted in the void volume. Each preparation was analyzed for liposome phosphorus content and for the amount of trapped radioactive marker.

[^{14}C]Sucrose release from toxin-treated liposomes. Aliquots of the liposomes were incubated with various concentrations of streptolysin S in a total volume of 0.3 ml. After 30 min, the toxin/liposome mixtures were applied to Sepharose 4B columns as

described above. Approx. 10 min was required for complete separation of intact liposomes from the released marker. The additional time that the liposomes were in contact with the toxin was estimated to be less than 4 min. Fractions (0.7 ml) were collected and 0.1 ml was added to 3a70B scintillation cocktail (Research Products International). Radioactivity was determined by scintillation counting. The cpm in the two resulting peaks were summed and the proportion of the total counts that was present in the second peak was used to indicate the amount of marker released by the toxin. For experiments in which the effect of temperature on toxin action was studied, the column was equilibrated and run at 4°C.

Toxin binding experiments. Liposomes (0–0.45 ml) were incubated with 50 hemolytic units of steptolysin S in a total volume of 0.5 ml for 15 min at 37°C. The samples were centrifuged at 23 000 X*g* for 15 min at 4°C and the titer of the hemolytic activity remaining in the supernatant was determined as described above.

Liposome charge. Liposomes bearing a net positive or negative charge were prepared by including 1.0 mol% stearylamine or 0.5 mol% dicetylphosphate in the lipid mixtures. Because liposomes containing stearylamine had a tendency to stick to the Sepharose columns, the liposomes used in the charge experiments (including uncharged liposomes) were separated from untrapped sucrose by centrifugation at 23 000 X*g* for 15 min. The pellet was washed twice in low salt buffer and resuspended in 2 ml of the same buffer. After treatment with streptolysin S for 30 min at 37°C, the samples were centrifuged again and the amount of label present in the supernatant was determined as described above.

Other procedures. Protein was assayed fluorometrically [16]. Phosphorus determinations were carried out with Fiske-SubbaRow reagent as described by Bartlett [17].

Results

Effect of streptolysin S on unilamellar vesicles

Liposomes composed of phosphatidylcholine, cholesterol and dicetylphosphate were incubated with various concentrations of streptolysin S at 37°C for 30 min. The [^{14}C]sucrose released by the toxin was separated from the label remaining entrapped in the liposomes by running the mixture on Sepharose 4B columns. The results of a typical experiment (Fig. 1) show that with increasing toxin concentrations, increasing amounts of the radioactive marker were liberated (fractions 5–8). Similar results were obtained when cholesterol was omitted from the liposomes. There was virtually no release of sucrose when trypan blue, an inhibitor of streptolysin S, was added to the reaction mixture.

Influence of liposome charge and phospholipid headgroup composition on streptolysin S action

The possibility that the net charge of the liposomes affects their susceptibility to streptolysin S was tested by including either dicetylphosphate or stearylamine in vesicles prepared with the uncharged lipid, phosphatidylcholine. The results (Fig. 2) demonstrated that liposomes bearing either a positive or negative surface charge were equally susceptible to the toxin and comparable to uncharged liposomes in that respect.

The effect of the composition of the hydrophilic

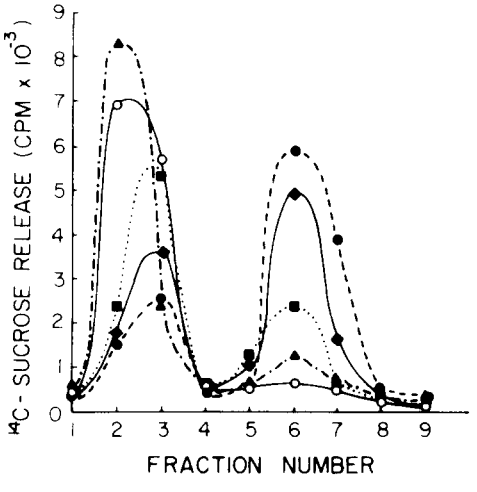

Fig. 1. Effect of streptolysin S on unilamellar liposomes composed of phosphatidylcholine, cholesterol and dicetyl phosphate in a molar ratio of 1.5 : 1.0 : 0.01. The liposomes, which contained [^{14}C]sucrose as a marker, were treated with various concentrations of streptolysin S for 30 min at 37°C. The samples were passed through a Sepharose 4B column to separate the sucrose released by toxin action (fractions 5–8) from that remaining trapped in the liposomes (fractions 1–4). Toxin concentrations in hemolytic units were 10 (▲); 25 (■); 50 (◆); 100 (●); 100 + 5 μg trypan blue (○).

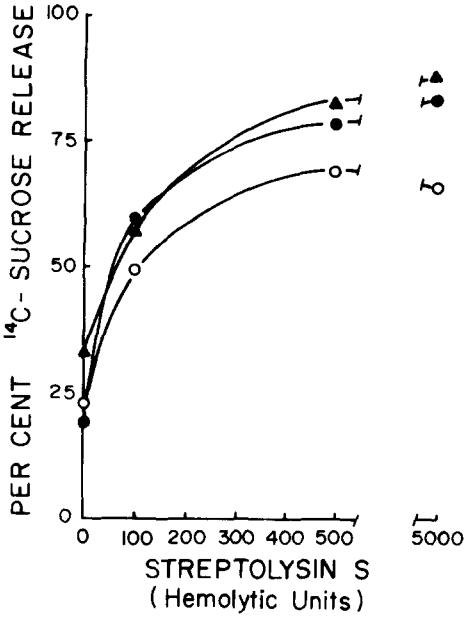

Fig. 2. Effect of liposome charge on streptolysin S action. Liposomes composed of phosphatidylcholine and cholesterol (○), phosphatidylcholine, cholesterol, and stearylamine (▲), or phosphatidylcholine, cholesterol and dicetyl phosphate (●), were incubated with various concentrations of toxin for 30 min at 37°C. The liposomes were pelleted by centrifugation and the amount of labeled marker in supernatant was determined.

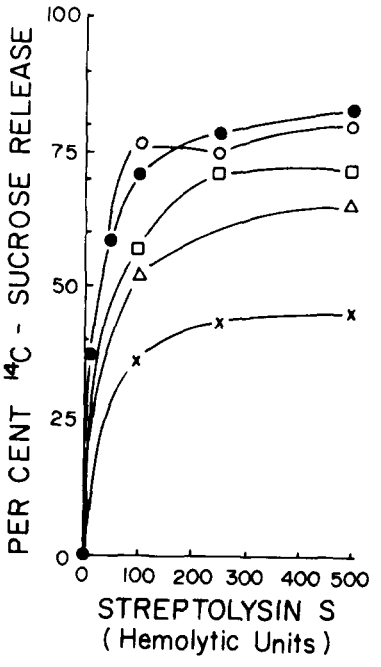

Fig. 3. Toxin effect on liposomes composed of various glycerophospholipids or sphingomyelin. The liposomes, which were incubated with streptolysin S for 30 min at 37°C, consisted of phospholipid + cholesterol + dicetyl phosphate in a molar ratio of 1.5 : 1.0 : 0.01. Phosphatidylcholine (●); phosphatidylethanolamine (○); phosphatidylglycerol (△); phosphatidylserine (□); sphingomyelin (X). The phosphatidylglycerol liposomes did not contain cholesterol.

region of the lipid molecules on toxin action was tested by preparing large unilamellar vesicles composed of phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol or phosphatidylserine. In addition, sphingomyelin, which contains a phosphocholine headgroup was tested in these experiments. All the glycerophospholipid liposomes were about equally susceptible to streptolysin S damage (Fig. 3). Sphingomyelin liposomes were also affected by the toxin, but the extent of membrane damage, as determined by the release of labeled sucrose, was markedly lower than that seen in phosphatidylcholine liposomes.

Effect of fatty acyl chain composition on toxin action

To examine the influence of the hydrophobic region of the phospholipid molecule, liposomes were prepared with C16 : 0 phosphatidylcholine, C18 : 0 phosphatidylcholine, C18 : 1 phosphatidylcholine or C18 : 2 phosphatidylcholine, and treated with various concentrations of streptolysin S at 37°C. Significant release of the sucrose marker was seen with C18 : 1 phosphatidylcholine and C18 : 2 phosphatidylcholine vesicles (Fig. 4A), but the toxin appeared to have no effect on liposomes composed of phosphatidylcholine containing saturated fatty acyl chains. However, when 40 mol% cholesterol was included in the liposomes, streptolysin S addition resulted in the release of significant levels of sucrose from C16 : 0 phosphatidylcholine and C18 : 0 phosphatidylcholine vesicles (Fig. 4B). Cholesterol also increased the sensitivity of C18 : 1 phosphatidylcholine vesicles to the toxin, but had little effect on the total amount of marker released from C18 : 1 phosphatidylcholine liposomes.

The experiments just described suggested that the physical state of the membrane may be important in

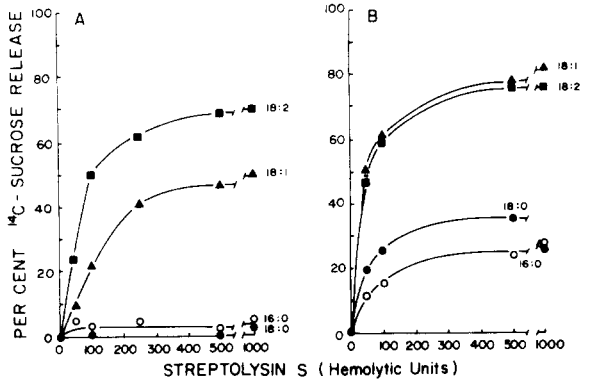

Fig. 4. Influence of phosphatidylcholine fatty acyl chain composition on streptolysin S activity. Liposomes consisting of C16 : 0, C18 : 0, C18 : 1, or C18 : 2 phosphatidylcholine plus dicetyl phosphate, without (A) or with 40 mol% cholesterol (B), were treated with streptolysin S and the amount of marker released was determined.

streptolysin S action. This idea was tested further by treating liposomes composed of C14 : 0 phosphatidylcholine with toxin at various temperatures above and below the chain-melting transition temperature (23°C) of this phospholipid. Separation of intact liposomes from released marker was carried out on a column equilibrated at 4°C. The results of two such experiments, shown in Fig. 5, indicate that streptolysin S had little or no effect on the liposomes at temperatures below the phase transition temperature, whereas there was an increasing release of sucrose above the transition temperature.

To test whether streptolysin S was able to bind to toxin-insensitive vesicles, liposomes composed of C18 : 0 phosphatidylcholine, C18 : 1 phosphatidylcholine or C18 : 2 phosphatidylcholine were incubated with 50 hemolytic units of toxin for 15 min at 37°C. The suspension were centrifuged to pellet the liposomes, and the amount of biologically active streptolysin S remaining was determined by incubating dilutions of the supernatant with erythrocytes at 37°C. There was a significant decrease in toxin activity after incubation with either C18 : 1 phosphatidylcholine or C18 : 2 phosphatidylcholine vesicles, but very little loss of activity after incubation with liposomes containing comparable amounts of C18 : 0 phosphatidylcholine (Fig. 6).

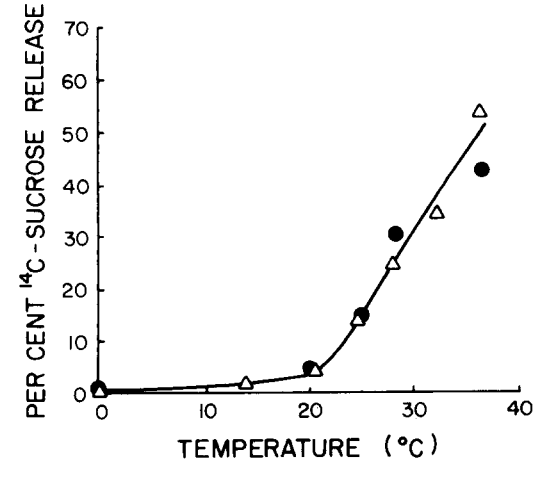

Fig. 5. Effect of streptolysin S on C14 : 0 phosphatidylcholine at various temperatures. The liposomes, consisting of phosphatidylcholine, cholesterol and dicetyl phosphate in a molar ratio of 2.3 : 0.2 : 0.01, were treated with 100 hemolytic units of toxin. Two experiments are shown (●, △).

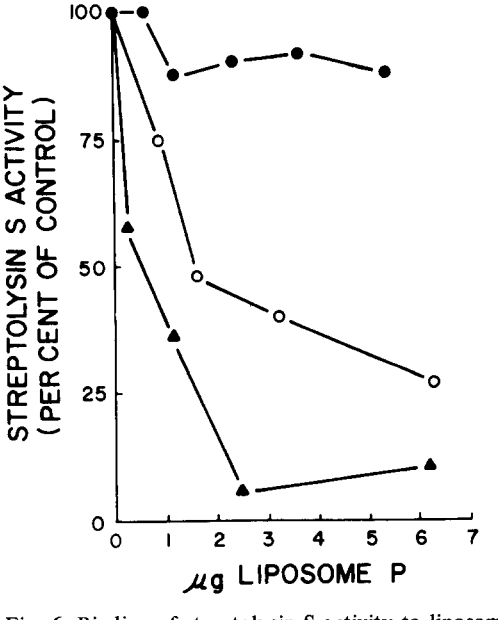

Fig. 6. Binding of streptolysin S activity to liposomes. Various concentrations of C18 : 0 (●), C18 : 1 (○), or C18 : 2 (▲) phosphatidylcholine + dicetyl phosphate were incubated with 50 hemolytic units streptolysin S for 15 min at 37°C. The liposomes were removed by centrifugation, and the hemolytic activity remaining in the supernatant was determined. Control tubes contained no liposomes.

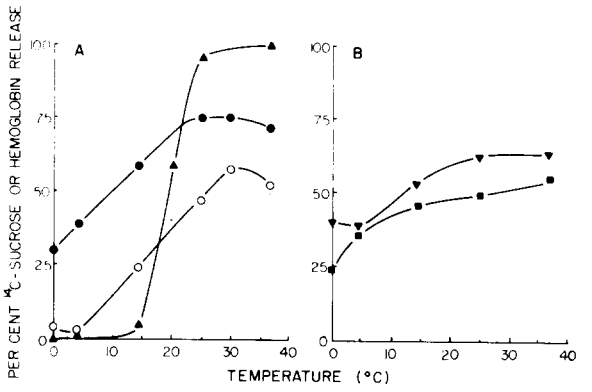

Fig. 7. Influence of temperature on streptolysin S action. (A) Erythrocytes (▲) or egg phosphatidylcholine-dicetyl phosphate liposomes, with (●) or without (○) 40 mol% cholesterol were incubated with 100 hemolytic units of toxin at various temperatures for 30 min. (B) C18 : 2 phosphatidylcholine-dicetyl phosphate vesicles with (▼) or without (■) 40 mol% cholesterol were treated with 100 hemolytic units streptolysin S. Each point represents the average of two experiments.

Influence of temperature on streptolysin S action

Erythrocytes treated with streptolysin S for 30 min at temperature below 10°C are unaffected by the toxin (Ref. 9 and Fig. 7A). Similarly, no release of [^{14}C]sucrose from toxin-treated egg phosphatidylcholine vesicles was seen at 0 or 4°C (Fig. 7A). However, when cholesterol (40 mol%) was included, the liposomes were found to be susceptible to membrane damage by the toxin at 0 or 4°C. There was an increase in the amount of label released at higher temperatures, with or without cholesterol, that was maximal at 25–30°C. Fig. 7B demonstrates that C18 : 2 phosphatidylcholine liposomes were sensitive to streptolysin S action at low temperatures in the presence or absence of cholesterol.

Discussion

Unilamellar liposomes produced by reverse-phase evaporation techniques appear to be extremely sensitive models for investigating the membrane-damaging effects of cytolytic toxins. Rosenqvist et al. [18] studied the effects of another streptococcal toxin, streptolysin O, by following the release of TEMPO-choline chloride from two types of liposome. Concentrations of streptolysin O that produced no effect on multilamellar vesicles caused rapid membrane damage and release of the internally trapped marker from large unilamellar vesicles. In experiments reported here, the addition of low concentrations of streptolysin S (100 hemolytic units; 0.2 μg protein) resulted in the release of 75–80% of internally trapped [^{14}C]sucrose from phosphatidylcholine vesicles (2–6 μg liposome phosphorus).

The net charge of the vesicles and the composition of the polar region of the phospholipid molecule appear to have very little effect on the ability of streptolysin S to produce membrane damage in unilamellar vesicles. The reduced sensitivity of sphingomyelin liposomes compared with phosphatidylcholine vesicles (Fig. 3) is presumably a reflection of the sphingosine or amide-linked fatty acid components of sphingomyelin, rather than the phosphocholine headgroup of the molecule.

In contrast to the hydrophilic regions of the phospholipid molecule, the composition of the fatty acyl chains appears to be very important in streptolysin S action. The ability of the toxin to produce membrane damage in phosphatidylcholine liposomes depended on the degree of unsaturation of the fatty acids; at 37°C, the order of sensitivity was C18 : 2 phosphatidylcholine > C18 : 1 phosphatidylcholine > C18 : 0 phosphatidylcholine = C16 : 0 phosphatidylcholine, with liposomes composed of the latter two phospholipids being virtually unaffected by streptolysin S. Experiments in which the loss of toxin activity was measured after incubation with the vesicles, indicated that streptolysin S did not bind to liposomes composed of phosphatidylcholine containing saturated fatty acyl chains. The suggestion that the nature of the hydrophobic region of the membrane is important in streptolysin S action was supported by the results of two additional experiments. First, the inclusion of high concentrations of cholesterol (40 mol%) in C16 : 0 phosphatidylcholine, C18 : 0 phosphatidylcholine and C18 : 1 phosphatidylcholine vesicles significantly increased the sensitivity of these liposomes to the toxin. Second, streptolysin S had little or no effect on C14 : 0 phosphatidylcholine at temperatures below the transition temperature of this phospholipid, but produced increasing membrane damage above the transition temperature.

Together, the data suggest that the fluidity of the phospholipid hydrocarbon chains in the membrane

may be important in streptolysin S action. Liposomes in which the phospholipids were presumably in a gel or crystalline array (C16 : 0 phosphatidylcholine and C18 : 0 phosphatidylcholine at all temperatures in these experiments; C14 : 0 phosphatidylcholine at temperatures below 23°C) were refractory to toxin action. However, when the physical state of the lipids was altered, by raising the temperature above the transition temperature, or by adding cholesterol, liposome membrane damage was readily observed. Both of these alterations would tend to disrupt to ordered array of the phospholipid hydrocarbon chains, increasing the fluidity of the membrane [19].

The influence of cholesterol on streptolysin S action deserves additional comment. Weissmann and Sessa [13] demonstrated that the release of CrO_4^{2-} from egg phosphatidylcholine-dicetylphosphate lipo somes was unaffected by the presence of cholesterol [13]. Our results support the idea that cholesterol, per se, is not directly involved in the action of this toxin. Significant membrane damage was produced by streptolysin S in the absence of cholesterol when the vesicles contained phosphatidylcholine with unsaturated fatty acyl chains. Nevertheless, vesicles composed of C16 : 0 phosphatidylcholine and C18 : 0 phosphatidylcholine, which were unaffected by high concentrations of streptolysin S, were made susceptible to toxin action by the inclusion of cholesterol. The simplest explanation is that cholesterol prevents the ordering and crystallization of the fatty acyl chains that occur below the transition temperature of these phospholipids. The inclusion of cholesterol also affected C18 : 1 phosphatidylcholine, and to a lesser extent, C18 : 2 phosphatidylcholine liposomes. These results probably reflect the modulating effect of cholesterol on the hydrocarbon chains of these lipids, which would have fluid properties at the temperatures used in these experiments. This may also account for the fact that cholesterol increased the susceptibility of egg phosphatidylcholine liposomes to streptolysin S at O and 4°C. The latter observation is somewhat puzzling, however, because erythrocytes, which contain comparable amounts of cholesterol in their membranes, were not affected by the toxin at low temperatures. Other membrane components, including proteins and glycolipids, may influence streptolysin S action at low temperatures.

One possible explanation for the colloid-osmotic nature of steptolysin S cytolysis [9,10] is that the toxin peptide is inserted into the lipid bilayer of the cell membrane. The peptides may then come together to form transmembrane channels, resulting in the flow of small molecules and water across the membrane. The results of our studies with unilamellar liposomes suggest that if this is indeed the case, peptide insertion and/or channel formation must occur in regions of the membrane where the fluidity of the phospholipid hydrocarbon chains is compatible with these processes.

Acknowledgement

This investigation was supported by U.S. Public Health Service Grant AI-11644 from the National Institute of Allergy and Infectious Diseases.

References

1 Ginsburg, I. (1970) in Microbial Toxins (Montie, T., Kadis, S. and Ajl, S., eds.), Vol. 3, pp. 99–171, Academic Press, New York
2 Jeljaszewicz, J., Szmigielski, S. and Hryniewicz, W. (1978) in Bacterial Toxins and Cell Membranes (Jeljaszewicz, J. and Wadstrom, T., eds.), pp. 185–227, Academic Press, London
3 Alouf, J.E. (1980) Pharmacol. Ther. 11, 661–717
4 Calandra, G.B. and Cole, R.M. (1981) Infect. Immunol. 31, 386–390
5 Bernheimer, A.W. (1967) J. Bacteriol. 93, 2 024–2 025
6 Lai, C.Y., Wang, M-T., De Faria, J.B. and Akao, T. (1978) Arch. Biochem. Biophys. 191, 804–812
7 Elias, N., Heller, M. and Ginsburg, I. (1966) Isr. J. Med. Sci. 2, 302–309
8 Okazaki, H. (1971) J. Biochem. (Tokyo) 70, 867–868
9 Duncan, J.L. and Mason, L. (1976) Infect. Immunol. 14, 77–82
10 Hryniewicz, W., Szmigielski, S. and Janiak, M. (1977) Zblt. Bakt. Hyg. I. Abt A 238, 201–207
11 Thelestam, M. and Mollby, R. (1975) Infect Immunol. 12, 225–232
12 Bangham, A., Standish, M. and Weissmann, G. (1965) J. Mol. Biol. 13, 253–259
13 Weissmann, G. and Sessa, G. (1967) J. Biol. Chem. 242, 616–625
14 Szoka, F. and Papahadjopoulos, D. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 4 194–4 198
15 Bernheimer, A.W. (1949) J. Exp. Med. 90, 373–392
16 Lai, C. (1977) in Methods in Enzymology (Hirs, C. and Timersheff, S., eds.), Vol. 47, pp. 236–243, Academic Press, New York
17 Bartlett, G.R. (1959) J. Biol. Chem. 234, 466–468
18 Rosenqvist, E., Michaelsen, T.E. and Vistnes, A.I. (1980) Biochim. Biophys. Acta 600, 91–102
19 Demel, R.A. and De Kruyff, B. (1976) Biochim. Biophys. Acta 457, 109–132