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INTERACTION BETWEEN SPHINGOMYELIN AND A CYTOLYSIN FROM THE SEA ANEMONE *STOICHACTIS HELIANTHUS*

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Summary

The cytolytic toxin from the sea anemone *Stoichactis helianthus* was inhibited up to 90—95% by suspensions of sphingomyelin but not by phosphatidylcholine or other membrane lipids. When the toxin was incubated with sphingomyelin and the mixture fractionated either by isoelectric focusing or Sephadex gel filtration, the residual hemolytic units migrated together with the lipid and not as free toxin. Incubation with phosphatidylcholine, however, did not shift the toxin peak in either type of column.

A toxin-ferritin conjugate retaining hemolytic activity was observed by negative staining to bind to liposomes prepared with sphingomyelin but not with liposomes containing phosphatidylcholine. The results provide evidence that the membrane binding site of the toxin is sphingomyelin.

Introduction

A hemolytic toxin, lethal to mice, was purified from the sea anemone *Stoichactis helianthus* by Devlin [1]. The toxin was further characterized in this laboratory [2] and found to be cytolytic to several kinds of mammalian cells in addition to erythrocytes, but not to bacteria or bacterial protoplasts. It is a protein of molecular weight 16 000 containing about 4% carbohydrate, and has an isoelectric pH of 9.8. It was found to be specifically inhibited by small amounts of sphingomyelin and by human serum, but not by other purified lipids encountered in erythrocyte membranes. Pretreatment of erythrocyte membranes with staphylococcal sphingomyelinase destroyed their ability to bind the toxin. The toxin did not itself appear to be a sphingomyelinase [2]. These properties suggested a similarity to a group of cytolytic toxins produced by bacteria which have been shown to be inhibited by membrane phospholipids or sterols [3].

If, as is thought [2,4,5], the specific lipids which inhibit such toxins are in fact their membrane binding sites, physical complexing between the purified toxins and their lipid inhibitors should be demonstrable. We have undertaken to show such a complex between *S. helianthus* toxin and sphingomyelin using standard methods of protein fractionation as well as electron microscopy of liposomes treated with ferritin labelled toxin.

Materials and Methods

Toxin. Purified hemolysin (F-4) from *Stoichactis helianthus* was generously supplied by Dr. John Devlin. It was prepared by a slight modification of his originally published procedure [1], and had a specific activity of 50 000 hemolytic units (HU) per mg against rabbit erythrocytes. Toxin used in the present study was not subjected to further purification except where indicated.

Preparation of lipid suspensions and liposomes. Lyophilized sphingomyelin (General Biochemicals, Chagrin Falls, Ohio) or DL-α-phosphatidylcholine (Sigma Chemicals Inc., St. Louis, Mo.) were taken up in 0.145 M NaCl/0.01 M Tris · HCl, pH 7.2 (Tris-saline) to a concentration of 1 mg/ml, and then subjected to sonication at 4° C until a homogeneous suspension was obtained (2—3 min). To prepare radioactively labelled sphingomyelin suspensions, the desired quantity of [*N-Me*-^{14}C]sphingomyelin, 60 Ci/mol (Radiochemical Center, Amersham, U.K.), in benzene-ethanol was dried under N_2 at the bottom of a 3 ml conical centrifuge tube. Sonicated, non-radioactive lipid suspension was added to the concentration required, and mixing was achieved by alternately treating in a sonic bath and vortexing. Estimation of radioactivity was done in a Nuclear Chicago Mark I scintillation counter using aqueous counting scintillation fluid from Clinical Assays, Inc. (Cambridge, Mass.).

Liposomes containing cholesterol (Sigma Chemicals Inc., St. Louis, Mo.), a charged amphiphile and a phospholipid were prepared according to Kinsky [6], except that displacement of the lipid film from the glass vessel was done in a sonic bath.

Preparation of helianthus toxin-ferritin conjugate. Horse spleen ferritin (6 times recrystallized, Cd-free, Miles-Pentex, Inc., Kankakee, Ill.) was further purified by recrystallization and ultracentrifugation as described by Andres et al. [7]. Conjugation of helianthus toxin (7 mg) to ferritin (38 mg) was done using toluene 2,4-diisocyanate (Polysciences, Inc., Rydal, Pa.) by the method of Singer and Schick [8]. The conjugate was washed 3 times by centrifugation at 100 000 × *g* until hemolytic activity could be detected in the supernatant. The pellet was dissolved in 2 ml of 0.05 M phosphate buffer, pH 7.5. The solution had a titer of 640 HU/ml.

Analytical procedures. Hemolytic activity was estimated as previously described [9] except that gelatin was omitted. 1 HU is defined as that amount of toxin producing 50% hemolysis when incubated with 1 ml of a 0.7% suspension of washed rabbit erythrocytes at 37° C for 30 min.

Inorganic phosphorus was estimated by the method of Ames and Dubin [10].

Electron microscopy. Samples of ferritin labelled liposomes in 0.05 M phosphate buffer, pH 7 were placed on Formvar-supported, carbon-coated grids.

Excess buffer was removed with a piece of filter paper and the samples were stained in 2% ammonium molybdate for 1 min.

Samples were examined in a Sieman's Elmiskop 1 equipped with a decontamination device and operated at 80 kV.

Results

Inhibition of hemolysis by sphingomyelin. In order to study the effect on hemolytic activity of incubation of helianthus toxin with increasing concentrations of sphingomyelin, aliquots of toxin containing 20 HU/ml were incubated with lipid suspensions for 1 h at 37° C, fast frozen and maintained in the frozen state until it was convenient to perform the hemolytic titration. The mixtures were thawed and diluted for estimation of hemolytic activity. As was previously reported [2], 2 μg/ml sphingomyelin reduced the activity by approximately two-thirds (Fig. 1). Increasing the sphingomyelin concentration further reduced the activity up to a concentration of approximately 15 μg/ml, but a residual 5—10% of hemolytic activity was maintained at concentrations of lipid up to 500 μg/ml. 500 μg/ml of sphingomyelin itself produced no lysis of erythrocytes.

Demonstration of specific binding of toxin to sphingomyelin by isoelectric focusing

Helianthus toxin has been shown to be isoelectric at about pH 9.8 [2]. Using the same conditions, isoelectric focusing of an aqueous dispersion of sphingomyelin resulted in a single peak centered at approximately pH 7 (Fig. 2). In this case sphingomyelin was localized by estimation of inorganic phosphorus in the fractions. However, the insoluble aqueous dispersion of lipid was visible as a turbid band in the center of the column several hours after the voltage was applied. Small portions of such lipid bands were sometimes observed to fall away from the major band and localize at lower positions in the column during the course of the focusing runs. Isoelectric focusing of toxin-sphingomyelin

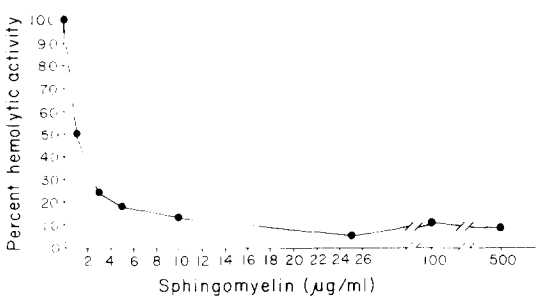

Fig. 1. Inhibition of hemolytic activity of helianthus toxin with increasing concentration of sphingomyelin. Incubation mixtures contained 20 HU/ml and the indicated concentration of sphingomyelin in a final volume of 5 ml. Incubation was at 37°C for 1 h, followed by fast freezing. Mixtures were thawed and diluted for estimation of hemolytic activity and results expressed as per cent of initial HU/ml.

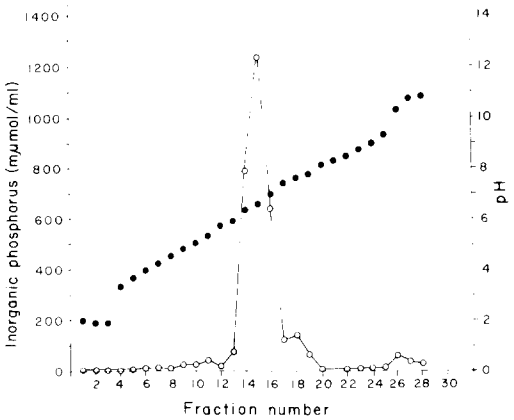

Fig. 2. Isoelectric focusing of sphingomyelin. 36 mg sphingomyelin sonicated to a uniform suspension in 48 ml distilled water was electrofocused for 46 h in a sucrose gradient using ampholines of pH 3—10 under the conditions in ref. 2. 4 ml fractions were collected and monitored for pH (●———●). An aliquot of each fraction was dialyzed extensively against distilled water before estimation of inorganic phosphorus (○———○).

mixtures was carried out using [^{14}C]sphingomyelin suspensions prepared as described in Materials and Methods. 4.5 mg helianthus toxin in 4.5 ml Tris-saline was mixed with 4 mg [^{14}C]sphingomyelin in 4 ml Tris-saline containing 25 nCi/mg. The mixture was incubated at 37°C for 1 h, a sample removed for estimation of hemolytic activity, and the remainder subjected to isoelectric focusing in a pH 3—10 gradient in the same fashion as the pure lipid. Visual observation of the column revealed a dense band of insoluble material in the center similar to that observed for pure sphingomyelin. Fractions having a volume of 3 ml were collected and monitored for absorbance at 280 nm, pH, hemolytic activity, and radioactivity (Fig. 3). Incubation of the toxin with sphingomyelin

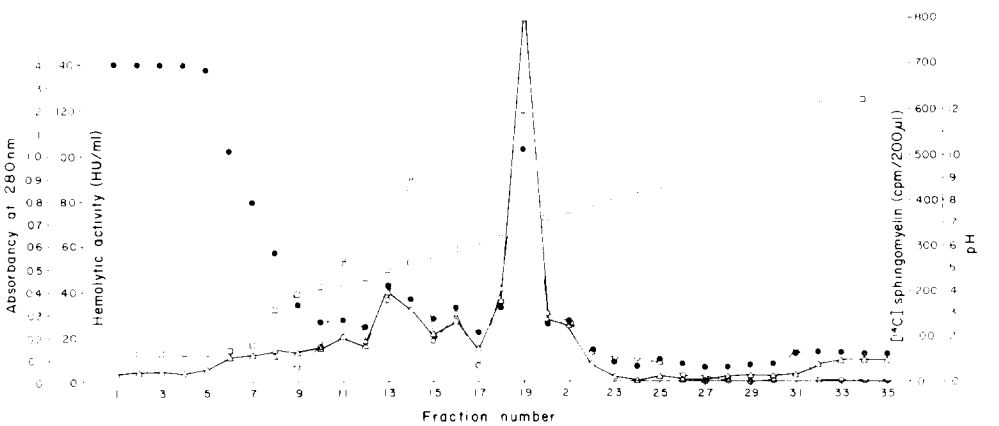

Fig. 3. Isoelectric focusing of helianthus toxin/[^{14}C]sphingomyelin mixture. 4.5 mg helianthus toxin was incubated with 4 mg [^{14}C]sphingomyelin containing 25 nCi/mg in a final volume of 8.5 ml Tris-saline at 37°C for 1 h. The mixture was subjected to isoelectric focusing as described in Fig. 2. Fractions of 3 ml were collected and monitored for pH (○———○), absorbancy at 280 nm (●- - - - -●), radioactivity (△———△), and hemolytic activity (○———○).

in this case produced a 96% inhibition of hemolytic activity. 64% of the activity was recovered from the column and almost all of it was found to be associated with radioactivity, with the major peak occurring at the position of free sphingomyelin (pH 7), and smaller peaks at lower pH values. The latter regions may be an artifact of the physical breaking apart of the major peak as described above, or may represent complexes with altered lipid-to-protein ratios. Only a very small number of hemolytic units migrated at the position of free toxin (fraction 31), and there was no significant 280 nm absorbance in this region. The toxin accounted for approximately 3.2 units of the 280 nm absorbing material that had been applied to the column. Much of the ultraviolet absorbing material associated with radioactivity was due to light scattering by the sphingomyelin suspension.

Phosphatidylcholine is not inhibitory to hemolysis by the toxin [2]. When 16 000 HU of toxin were incubated with 2.5 mg phosphatidylcholine (non-radioactive) for 1 h at 37°C, no inhibition of hemolytic activity was observed. The incubation mixture was subjected to isoelectric focusing under the usual conditions, and the column fractionated into 3 ml portions. As with sphingomyelin, the lipid formed a dense band in approximately the center of the column. Fractions were monitored for pH, inorganic phosphorus and hemolytic activity. The results are shown in Fig. 4. 92% of the recovered hemolytic activity was localized in a peak at pH 9—10, the position to which free toxin migrates. No phosphorus could be detected in this region. The major part of the phospholipid focused sharply at pH 6.5 and was associated with 250 HU of toxin. The latter HU were normally sensitive to inhibition by sphingomyelin. The results obtained by electrofocusing demonstrate that helianthus toxin forms a physical complex with sphingomyelin whereby its activity is inhibited. In contrast, helianthus toxin does not form such a complex with phosphatidylcholine nor is it inhibited by this lipid.

Demonstration of specific binding of toxin to sphingomyelin by gel filtration

Binding experiments using gel filtration were done using toxin which was

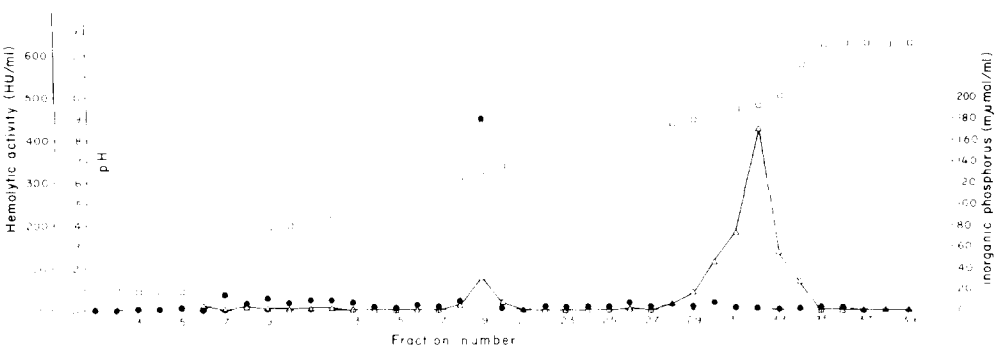

Fig. 4. Isoelectric focusing of helianthus toxin/phosphatidylcholine mixture. 1 mg helianthus toxin was incubated with 2.8 mg phosphatidylcholine in a final volume of 3.8 ml Tris-saline at 37°C for 1 h. The mixture was subjected to isoelectric focusing as described in Fig. 2, and 3 ml fractions collected. Fractions were monitored for pH (□———□) and hemolytic activity (△———△). Inorganic phosphorus (●———●) was measured on aliquots of each fraction which had been extensively dialyzed against distilled water.

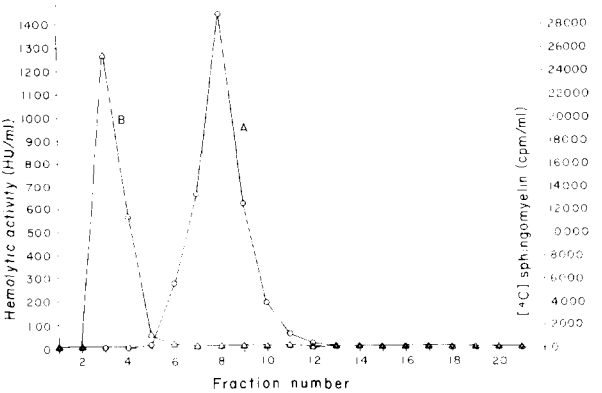

Fig. 5. Sephadex G-100 chromatography of helianthus toxin and [^{14}C]sphingomyelin. Curve A. 5000 HU of electrofocused helianthus toxin in 400 μl Tris-saline was applied to a column (19 X 0.9 cm) of Sephadex-G 100 equilibrated in Tris-saline. Elution was carried out with the same buffer and fractions of 1.4 ml collected and monitored for hemolytic activity (○———○). Curve B. 100 μg [^{14}C]sphingomyelin (0.1 μCi) in 100 μl Tris-saline was applied to the same column as in curve A, and eluted and collected in the same fashion. Fractions were monitored for radioactivity (△———△).

purified by isoelectric focusing [2]. 5000 HU of toxin was applied to a column (19 X 0.9 cm) of Sephadex G-100 equilibrated in Tris-saline and pretreated with bovine serum albumin. The exclusion volume as determined using blue dextran was 4.5 ml. Elution was carried out with Tris-saline, and 1.4 ml fractions collected. Fractions were monitored for hemolytic activity and the results shown in Fig. 5, curve A. A single peak of activity was centered at tube 8. [^{14}C]sphingomyelin (100 μg containing 0.1 μCi) was run by itself on the same column. Approximately one half of the counts applied were eluted at the exclusion volume (Fig 5, curve B), and one half permanently bound to the gel. The same quantities of toxin and [^{14}C]sphingomyelin were mixed, incubated at 37°C for 1 h, sampled for estimation of hemolytic activity, and again applied to the column, and eluted in the same way. Incubation with lipid in this case produced 98% inhibition of hemolytic activity, but as can be seen from Fig. 6,

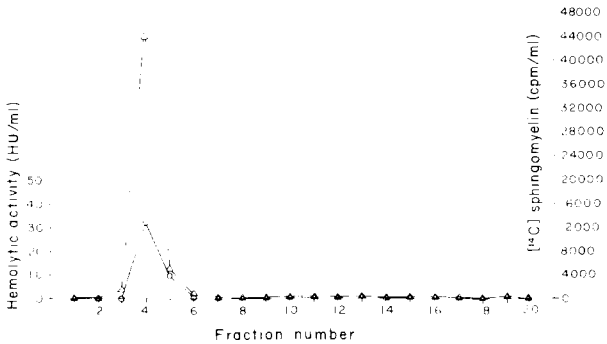

Fig. 6. Sephadex G-100 chromatography of helianthus toxin/[^{14}C]sphingomyelin mixture. 5000 HU of electrofocused helianthus toxin + 100 μg [^{14}C]sphingomyelin (0.1 μCi) were incubated together for 1 h at 37°C in a final volume of 500 μl Tris-saline. The column was eluted and fractionated as in Fig. 5. Fractions were monitored for hemolytic activity (○———○), and radioactivity (△———△).

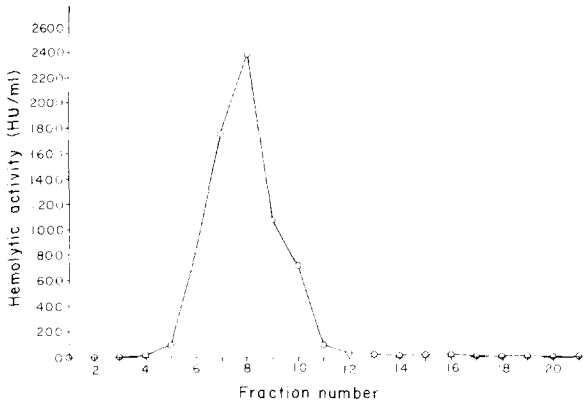

Fig. 7. Sephadex G-100 chromatography of helianthus toxin/phosphatidylcholine mixture. 12 000 HU of electrofocused toxin was incubated with 100 μg phosphatidylcholine at 37°C for 1 h in a final volume of 500 μl Tris-saline. The column was eluted and fractionated as in Fig. 5, and the fractions monitored for hemolytic activity.

the residual hemolytic units were eluted at the exclusion volume, coincident with the radioactive peak. A similar experiment was done using 2 mg of toxin not further purified by electrofocusing. This material has 1.5 units of 280 nm absorbing material, enough to be readily detected if the inactivated toxin were eluted at the position of free toxin. Virtually all of the protein recovered was in the excluded peak together with the [^{14}C]sphingomyelin (data not shown).

Phosphatidylcholine (0.1—1 mg) when applied to the Sephadex G-100 column always became irreversibly bound to the gel. 12 000 HU of electrofocused toxin were incubated with 100 μg of non-radioactive phosphatidylcholine, producing no inhibition of hemolytic activity. The hemolytic profile produced when this mixture was applied to the column can be seen in Fig. 7. The recovered toxin (85% of the HU applied) migrated in the position of free toxin, while the lipid remained attached to the gel. In a manner similar to the electrofocusing experiments, therefore, helianthus toxin bound to and fractionated together with the sphingomyelin aggregate, but not with phosphatidylcholine.

Binding of ferritin labelled helianthus toxin to liposomes

Two batches of liposomes were prepared according to the procedure described in Materials and Methods. Preparation A contained sphingomyelin, cholesterol and dicetylphosphate; preparation B had phosphatidylcholine, cholesterol and dicetylphosphate. The presence of these lipids in the liposomes was established by total lipid extraction followed by silica gel paper chromatography and visualization with iodine vapors. 0.3 ml of each type of liposome preparation in 0.05 M phosphate buffer, pH 7 was mixed with 50 μl of toxin-ferritin conjugate (32 HU) and incubated at 37°C for 45 min. Following incubation, the mixtures were diluted with 7 ml cold phosphate buffer and washed four times by centrifugation at 27 000 × *g* for 20 min.

Fig. 8A shows an electron micrograph of a negatively stained preparation of liposomes containing sphingomyelin and cholesterol which were treated with the ferritin-helianthus toxin conjugate. Heavy labelling was observed over the

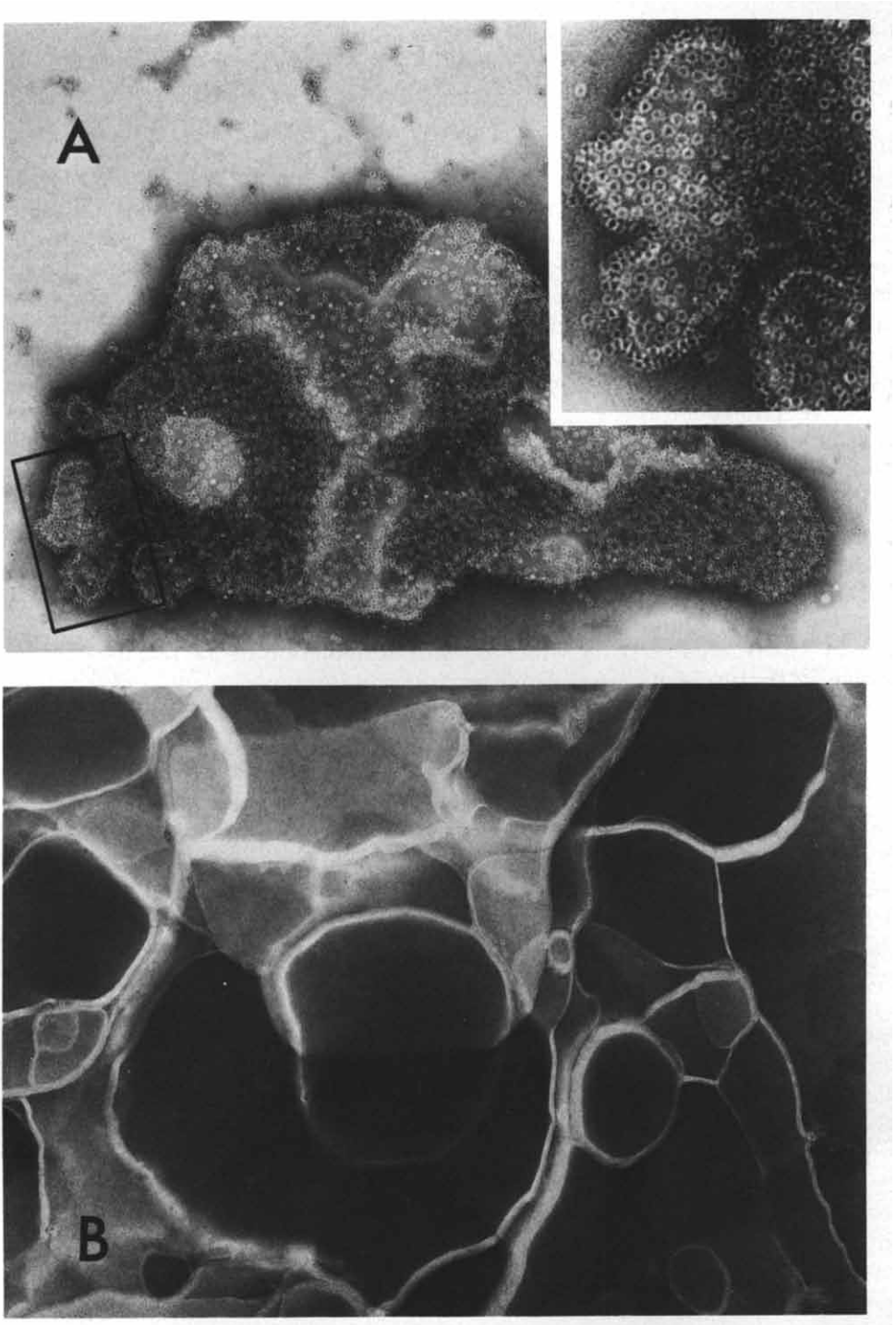

Fig. 8. Negatively stained liposomes treated with ferritin-helianthus toxin conjugate. A. Liposomes prepared with sphingomyelin and cholesterol were incubated with ferritin-conjugated helianthus toxin and washed. Samples were stained with 2% ammonium molybdate. Magnification ×72 000. The insert is a higher magnification of the area in the rectangle, magnification ×162 000. B. Liposomes prepared with phosphatidylcholine and cholesterol were incubated with ferritin-conjugated helianthus toxin and washed. Staining was as in A. Magnification, ×72 000.

surface of the vesicles and along their periphery (Fig. 8A insert). This is in contrast to liposomes containing phosphatidylcholine and cholesterol but not sphingomyelin (Fig. 8B) where only an occasional clump of ferritin was seen.

Discussion

Aqueous suspensions of sphingomyelin inhibit up to 90—95% of the hemolytic activity of *S. helianthus* toxin. Complete neutralization of activity was not obtained no matter how large the amount of lipid in the incubation mixtures. This is in contrast to the oxygen labile cytolysins produced by some bacteria, which are completely inhibited by small amounts of the appropriate sterols [4]. Such a pattern of incomplete inhibition is similar to that observed for virus neutralization by homologous antibody [11]. In the viral system, this is thought to reflect antibody molecules on some of the virus particles binding near, but not at the host cell binding site. This would then sterically prevent the binding of neutralizing antibody.

Unlike the viral system, the inhibitor in the case of helianthus toxin is not soluble in aqueous media, but rather exists in turbid suspension as aggregates or liquid crystals [12]. Because of the physical state of the sphingomyelin, it is difficult to speculate on the molecular nature of the toxin-lipid interaction, and any comparison with purely soluble systems should be considered cautiously.

It was this residual activity which permitted detection of the toxin-inhibitor complex in the present study. When protein and lipid were incubated together and the mixture fractionated by either electrofocusing or Sephadex gel filtration, the toxin migrated together with the sphingomyelin to the same locus as lipid alone. The residual hemolytic activity of the complex was used to localize the largely inactivated toxin. The absence of 280 nm absorbancy at pH 9—10 of the electrofocusing gradients, or in the included volume of the Sephadex gel ruled out the possibility that inactivated toxin dissociated from lipid and migrated as free protein. This is consistent with the findings of Hessinger and Lenhoff [13] who found that the hemolytic venom from the sea anemone *Aiptasia pallida*, even following inactivation by freezing and thawing, bound irreversibly to erythrocytes, and prevented binding by fresh toxin.

Incubation with phosphatidylcholine produced no inhibition as previously reported [2], and caused no shifting of the toxin peak in either type of column. In the case of the gel filtration column where the phosphatidylcholine could not be recovered from the gel, virtually all of the hemolytic units applied to the column were eluted in the usual toxin position. There was no evidence of protein adhering to the gel.

It appears that the toxin molecules become attached to the large sphingomyelin aggregates, rather than sequestering monomeric lipid molecules from them. This is indicated especially by the gel filtration experiments in which the toxin (molecular weight 16 000, see ref. 2) is eluted at the exclusion volume following incubation with sphingomyelin. This eliminated the possibility of drawing conclusions about the molecular weight of the complex or the stoichiometry of binding.

The roughly 5—10% of residual hemolytic activity remaining with the inhibited complex does not appear to represent contamination of the toxin by

another hemolytic agent. It is present both before and after electrofocusing of the Devlin (F-4) preparation and, more importantly, it shows specific affinity for sphingomyelin. It would seem reasonable to expect that sphingomyelin in the red cell membrane is in an environment better suited to binding toxin than is an artificial lipid crystal. If in fact the membrane lipid has a greater affinity for helianthus toxin that the pure lipid, one could possibly explain the residual hemolytic activity of the complex. A small portion of the toxin molecules, bound more loosely to the lipid aggregate, might be displaced relatively easily by the erythrocytes.

Prigent and Alouf [14] have recently reported that streptolysin O forms a visible halo in agar gels in which inhibitory sterols have been incorporated. The halo is interpreted by them to represent the insoluble complex between toxin and lipid, because of the requirement for stereospecific sterols. These results are similar to ours in so far as helianthus toxin becomes attached to the insoluble lipid aggregate.

Ferritin labelled *S. helianthus* toxin binds to liposomes containing sphingomyelin but not phosphatidylcholine, and this is in keeping with the inhibition and fractionation studies. Interaction of non-enzymatic cytolytic bacterial toxins with liposomes has been demonstrated in a number of systems [15–18], where release of small molecules or physical disorganization of the liposomes has been observed.

The direct observation of binding of hemolytically active ferritin-toxin conjugates specifically to sphingomyelin-containing liposomes, together with the biochemical identification of the toxin-lipid complex, further support the identification [2] of sphingomyelin as the membrane binding site for helianthus toxin. The present results are consistent with the previous suggestion as to the mechanism of hemolysis by this toxin [2]. The non-enzymatic protein binds to sphingomyelin in the outer half of the membrane bilayer [19] leading to disorientation of this component, producing permeability changes, and finally lysis [2].

Acknowledgements

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