Inhibition of *Staphylococcus aureus* Delta Hemolysin by Phospholipids[1] (36812)

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Assay of the individual *Staphylococcus aureus* hemolysins when present together has been difficult, with success frequently being dictated by their relative concentrations in such mixtures. Utilization of specific antisera permitted the selective neutralization of either alpha or beta hemolysin, but this approach did not suffice when delta hemolysin was also present. Lack of a neutralizing antibody against the delta hemolysin, together with its ability to lyse erythrocytes from different species with similar ease, precluded assay of the other hemolysins when they were present in low titer.

Most human strains of *S. aureus*, however, produce only the alpha and delta hemolysins. While conducting studies on the synthesis of alpha hemolysin in such strains it became necessary to measure low levels of this toxin in the presence of appreciable amounts of delta hemolysin. This situation initiated a search for an inhibitor of delta hemolysin and culminated with the demonstration that egg lecithin satisfied this purpose.

Materials and Methods. The alpha and delta hemolysins were produced, purified, and assayed as previously described (1). Only the soluble form of delta hemolysin was used in these studies. Crude culture supernatant fractions containing delta hemolysin and relatively small amounts of alpha hemolysin were prepared by growing *S. aureus* 18Z (2) in trypticase soy broth (BBL) under an O_2 atmosphere.

Kinetics of hemolysis were followed by measuring loss of turbidity of either a 0.1 or 1.0% washed rabbit erythrocyte suspension in saline at 660 nm using a recording spectrophotometer. Cuvettes, containing 4 ml reaction mixture, were maintained at 37° during the process. With this technique turbidity readings did not equate directly to lysis; for example, 50% lysis corresponded to 0.68–0.70 OD (20–22% *T*) at 660 nm.

Reagents were obtained from the following sources: phosphatidic acid and cardiolipin (beef heart) from General Biochemicals; phosphatidyl choline (synthetic), phosphatidyl ethanolamine (synthetic), phosphatidyl serine, sphingomyelin (beef heart) and phosphocholine from Mann Research Labs; dipalmitin, tripalmitin, palmitic acid, and egg lecithin (crude) from Nutritional Biochemicals; myristic acid and stearic acid from Delta Chemical Works; phosphatidyl inositol from L. Light & Co.; palmitoleic acid from K & K Labs; and staphylococcal antitoxin from Connaught Labs. Synthetic cardiolipin (palmitoyl) was prepared and supplied by Dr. Robin Saunders.

Crude egg lecithin was purified by adsorption onto silicic acid in chloroform followed by elution with chloroform–methanol mixtures of increasing methanol concentration. Purity of the final product was confirmed by thin-layer chromatography. Portions of purified egg lecithin were catalytically reduced with hydrogen in the presence of platinum black.

Lipids were dissolved in ethanol, rapidly diluted in saline before use, and appropriate amounts of the resulting emulsion were added to the test system. Care was taken that the final ethanol concentration did not exceed 2%. Controls without lipids, but containing equal amounts of ethanol were included.

Results. Purified egg lecithin when mixed with delta hemolysin at 1–2 μg/hemolytic unit (HD_{50}) completely inhibited its hemolytic activity toward human and rabbit erythrocytes. Much larger amounts of lecithin

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TABLE I. Inhibition of *S. aureus* Delta Hemolysin by Phospholipids.

Inhibitors[a]	Inactive[b]
Phosphatic acid (natural)	Myristic acid
Phosphatidyl choline (egg)	Palmitic acid
Phosphatidyl serine (natural)	Palmitoleic acid
Phosphatidyl inositol (natural)	Stearic acid
	Dipalmitin
Cardiolipin (beef heart)	Tripalmitin
Sphingomyelin (beef heart)	Choline
	Phosphocholine
	Glycerol
	Glycerol phosphate
	Phosphatidyl choline (synthetic)
	Phosphatidyl choline (egg, catalytically reduced)
	Phosphatidyl ethanolamine (synthetic)
	Cardiolipin (synthetic)
	Albumin (human serum)
	Cholesterol

[a] Active at 1–5 $\mu g/HD_{50}$.

[b] Inactive at 50–100 $\mu g/HD_{50}$ except for albumin and cholesterol, which were tested and found inactive at 10 mg/HD_{50}.

(100 $\mu g/HD_{50}$) had no effect upon partially purified alpha hemolysin. Other phospholipids of natural origin were also capable of inhibiting delta hemolysin at similar concentrations (Table I). A number of compounds, representing various moieties of the lecithin molecule, demonstrated no neutralizing activity. Synthetic phospholipids and egg lecithin, in which the fatty acids were saturated by reduction, also failed to inhibit delta hemolysin. Human serum albumin, previously reported to inhibit delta hemolysin (3), and cholesterol were found inactive at ratios of 10 mg/HD_{50}.

One international unit (IU) antitoxin neutralized 930 HD_{50} alpha hemolysin, but 1 IU/HD_{50} delta hemolysin caused no inhibition. By adding either antitoxin or lecithin to samples containing both alpha and delta hemolysin selective inactivation of one hemolysin was possible and the other could be measured. When assayed with rabbit erythrocytes the titer of such a mixture was determined by the hemolysin contributing the greatest hemolytic activity. No synergism affecting the titer was noted, yet a synergistic effect on the kinetics of hemolysis was observed (Fig. 1). With appropriate proportions of alpha and delta hemolysins a biphasic lytic response was noted. This could be resolved into a rapid, but limited phase of lysis due to delta hemolysin and a delayed, but more extensive lysis caused by the alpha hemolysin. The kinetics of lysis observed in the presence of lecithin or antitoxin resemble closely those reported for the alpha and delta hemolysins (3, 4).

Lecithin added to delta hemolysin–erythrocyte mixtures at various times during the lytic process rapidly halted further lysis (Fig. 2). Some inhibition was still evident even though lecithin was added after hemolysis was almost complete.

Discussion. Only natural phospholipids were found to inhibit delta hemolysin. This suggested the importance of unsaturated fatty acids in the molecule; a concept supported by the observation that egg lecithin reduced

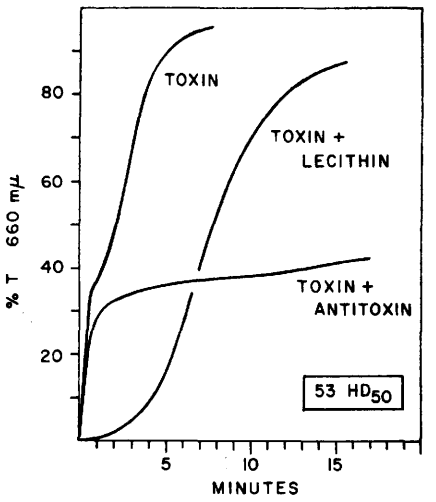

FIG. 1. Lysis of 1.0% washed rabbit erythrocyte suspension at 37° by culture supernatant fraction containing both alpha and delta hemolysins (toxin). The same preparation was rerun after adding (per ml) 1 IU of staphylococcal antitoxin (to neutralize alpha hemolysin) or 0.1 mg egg lecithin (to inhibit delta hemolysin). The original mixture contained (per ml) 53 HD_{50} alpha hemolysin and 8 HD_{50} delta hemolysin.

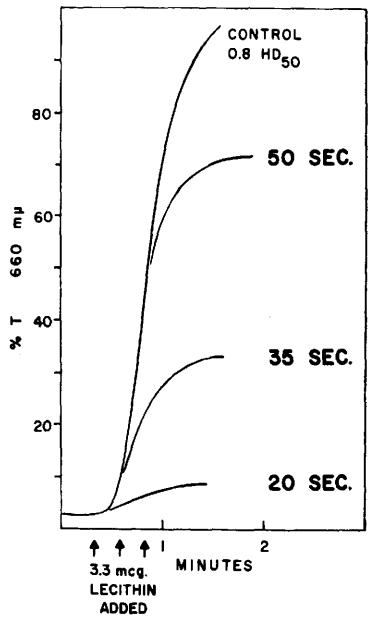

FIG. 2. Lysis of 0.1% washed rabbit erythrocyte suspension at 37° by purified delta hemolysin (control). The same preparation was rerun, but 3.3 μg egg lecithin added to the reaction mixture either 20, 35 or 50 sec after delta hemolysin.

with hydrogen no longer inhibited delta hemolysin. The minimal structural unit causing inhibition appeared to be phosphatidic acid and other components attached to the phosphate group did not significantly augment or diminish efficacy. Sphingomyelin, which resembles lecithin except for the sphingosine side chain, was also inhibitory.

Phospholipids did not appear to alter delta hemolysin *per se*. The addition of the nonhemolytic *B. cereus* phospholipase C to a neutralized mixture of delta hemolysin and egg lecithin fully restored the hemolytic activity as the lecithin was cleaved. Other studies (to be reported elsewhere) suggest that phospholipids inhibit delta hemolysin by interfering with its binding to the erythrocyte membrane.

A number of individuals have reported the anti-delta hemolysin activity of normal serum or serum proteins (3, 6 7) and the inability to increase this inhibitory activity with immunization (8). The phospholipid content of serum or crude protein fractions could readily account for this degree of neutralizing activity. Evidence that delta hemolysin is antigenic is currently unavailable.

Wiseman and Caird (9) have reported phospholipase activity associated with delta hemolysin. Although we found phospholipase activity present in crude culture filtrates, purified delta hemolysin (both soluble and insoluble forms) had no such ability. Nonhemolytic mixtures of phospholipids and delta hemolysin did not change after 24 hr at 37° whereas hemolytic activity was rapidly reinstated in the presence of *B. cereus* phospholipase C. The report by Wiseman and Caird does not provide sufficient data to estimate purity of their product, but findings of Kreger *et al.* (10) also fail to substantiate phospholipase activity in purified delta hemolysin preparations.

These studies also demonstrated a synergism between the alpha and delta hemolysin. This is evidenced primarily as an abolition of the lag phase asociated with alpha hemolysin activity. This synergism does not influence the titer of a preparation containing these products as is the case with beta–delta hemolysin mixtures.

Summary. A number of unsaturated phospholipids were found to be selective inhibitors of *Staphylococcus aureus* delta hemolysin. This inhibitory effect was manifest promptly after addition to delta hemolysin.

A synergism between alpha and delta hemolysin was evident in the kinetics of rabbit erythrocyte lysis.

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