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CHOLESTEROL-DEPENDENT TETANOLYSIN DAMAGE TO LIPOSOMES

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Summary

Tetanolysin caused membrane damage, resulting in release of trapped glucose from liposomes containing cholesterol. Maximum glucose release occurred from liposomes that contained 50 mol% cholesterol. At higher or lower levels of cholesterol, glucose release was reduced and glucose release did not occur at all below 40 mol% cholesterol. The apparent activity of tetanolysin was not influenced by temperature (24°C compared to 32°C) or by liposomal phospholipid fatty acyl chain length. We conclude that tetanolysin caused cholesterol-dependent lysin-mediated damage to liposomes, possibly by means of a pore consisting of a complex of toxin and cholesterol.

Tetanolysin is a cytolytic toxin produced by *Clostridium tetani*. It is a member of a group of closely related bacterial toxins that include streptolysin O, cereolysin, and at least twelve others [1,2]. Although the membrane receptor for all of the oxygen-labile toxins is cholesterol [1—3], and it has been proposed that all of the toxins have a common mode of action [1,2], the mechanism of toxin-induced membrane damage and permeability still is not completely clear. Interaction of oxygen-labile toxins with erythrocyte membranes or liposomes results in rings and arcs on the membrane surface as viewed by electron microscopy [4—6]. The rings caused by the toxins probably represent either aggregates of toxin or toxin-cholesterol complexes but they may or may not be the sites of pore formation [5,6]. Despite the appearance of rings

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on cholesterol-containing liposomes following exposure to streptolysin O [5,6], it has been claimed that the interaction of this toxin with liposomes does not cause increased permeability to glucose [5]. In contrast, a recent report demonstrated cholesterol-dependent glucose release from liposomes after incubation with cereolysin [7]. In the present study we investigated the question of lysin-mediated marker release from liposomes in more depth, and examined the influence of tetanolysin on the permeability of glucose from liposomes containing different quantities of cholesterol.

Tetanolysin was purified from crude culture filtrates of *C. tetani* using ammonium sulfate fractionation, gel filtration, and chromatography on hydroxyapatite. A single preparation of tetanolysin was used in all experiments. On discontinuous gel electrophoresis two bands were visualized, only one of which contained hemolytic activity. The other band was a protease. Using a previously described bioassay technique [8] the preparation contained only a trace contamination with tetanus neurotoxin ($2 \cdot 10^{-6}$ mg/mg tetanolysin). The preparation contained $1.8 \cdot 10^6$ hemolytic units of lysin per mg protein, and $5.4 \cdot 10^6$ hemolytic units per ml. Preparations were measured for specific activity by the visual method [9]. The definition of a hemolytic unit is arbitrary. In order to compare our data with that of previous investigators, we separately determined that one of our hemolytic units was equivalent to 30 hemolytic units as performed by the methods of Duncan and Schlegel [5] and Cowell and Bernheimer [7]. All of our expressions of hemolytic units have been multiplied by a factor of 30.

The preparation of liposomes and assay of release of trapped glucose are described in detail elsewhere [10]. The liposomes consisted of phospholipid, dicetyl phosphate, and cholesterol (where present). Phospholipid and dicetyl phosphate always were in molar ratios of 1 : 0.11. Calculation of mol% of cholesterol is based only on comparison with the phosphatidylcholine. The lipid combinations are given in the individual figures. The liposomes were swollen in 0.308 M glucose, and the phospholipid was 10 mM with respect to the final aqueous suspension. In a typical experiment, the toxin was added to a cuvette containing glucose assay reagent, and a baseline absorbance ($A_{340\ nm}$) was measured. The liposomes then were added on a plastic plumper. Increased $A_{340\ nm}$ was measured 30 min later and corrected for absorbance due to untrapped glucose and light scattering caused by the added liposomes [10].

Fig. 1 demonstrates that tetanolysin caused glucose release from liposomes, and the degree of damage was strongly dependent on cholesterol concentration. Glucose release did not occur at less than 40 mol% cholesterol, and maximum damage, resulting in almost 90% release of trapped glucose, occurred at 50 mol% cholesterol. At higher levels of cholesterol glucose release was diminished. It should be noted that in another system (cholesterol-dependent complement damage to liposomes) glucose release occurred only from liposomes containing more than 50 mol% cholesterol [10].

The dose-response of glucose release based on tetanolysin concentration is shown in Fig. 2. The total extent of glucose release and the apparent activity of tetanolysin were strongly dependent on cholesterol concentration (Fig. 2). At 50 mol% cholesterol the activity was not influenced by temperature (Fig. 2). The activity of tetanolysin was essentially the same when the liposomal phos-

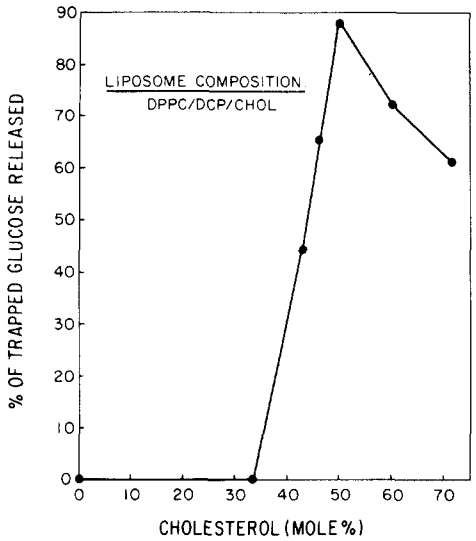

Fig. 1. Effect of cholesterol concentration on tetanolysin damage to liposomes. The liposomes (5 μl) having the indicated lipid composition were incubated at 24° C with 6000 hemolytic units of tetanolysin in a total volume of 1.0 ml. DPPC, dipalmitoyl phosphatidylcholine; DCP, dicetyl phosphate, CHOL, cholesterol.

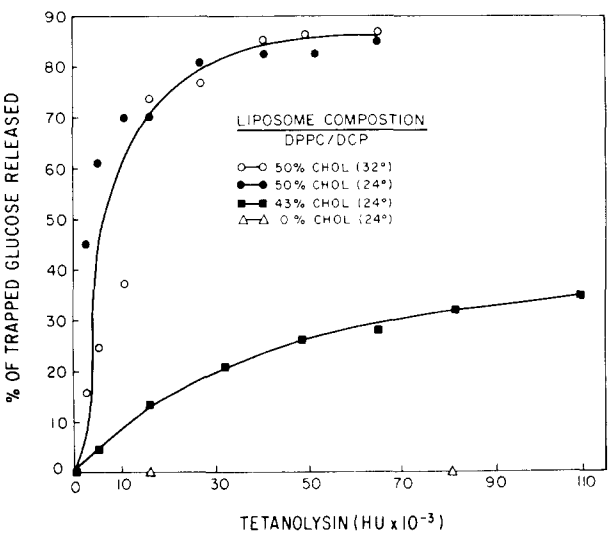

Fig. 2. Effect of cholesterol concentration and temperature on tetanolysin requirement for glucose release. Liposomes (5 μl) having the indicated lipid composition were incubated with tetanolysin at the indicated temperature in a total volume of 1.0 ml. DPPC, dipalmitoyl phosphatidylcholine; DCP, dicetyl phosphate; CHOL, cholesterol; HU, hemolytic units.

pholipid consisted of dimyristoyl, dipalmitoyl, or distearoyl phosphatidylcholine (Fig. 3).

In a previous study, Duncan and Schlegel [5] reported that streptolysin O did not cause glucose release from liposomes containing cholesterol, while Cowell and Bernheimer [7] found, as did we, that glucose release did occur from such liposomes in the presence of other oxygen-labile toxins. We believe that the apparent discrepancy between these studies is due to different experi-

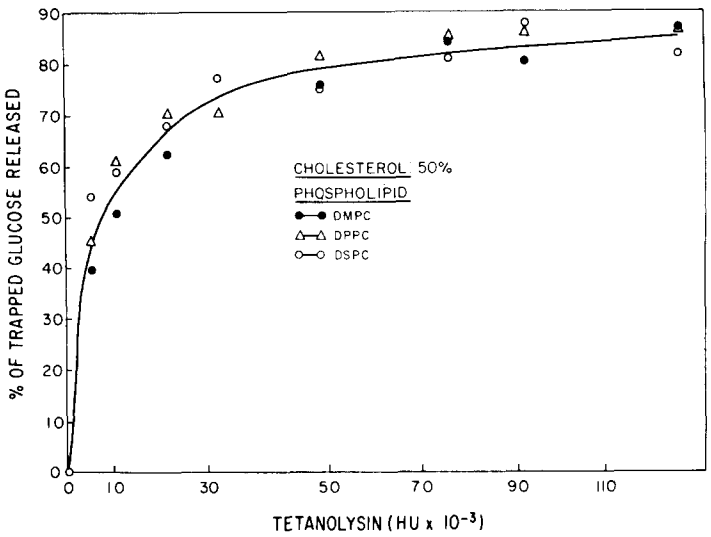

Fig. 3. Effect of phospholipid fatty acyl composition on tetanolysin damage. The liposomes (5 μl) were incubated with tetanolysin at 24° C in a total volume of 1.0 ml. DMPC, DPPC and DSPC; dimyristoyl, dipalmitoyl, and distearoyl phosphatidylcholine, respectively; HU, hemolytic units.

mental conditions rather than to different modes of action of the toxins. Based on an estimated molecular weight of 667 for egg phosphatidylcholine (derived from our own experimental measurements) we calculate that Duncan and Schlegel [5] used liposomes containing approx. 46 mol% cholesterol. They incubated 800 hemolytic units of streptolysin O with liposomes containing approx. 22.5 μmol phosphatidylcholine, giving a toxin/phospholipid ratio of 35.5 hemolytic units/μmol. In contrast, in Fig. 2 we show that 5400 hemolytic units of tetanolysin per 0.05 μmol phosphatidylcholine (toxin/phospholipid = 180 000 hemolytic units/μmol) caused about 45% glucose release from liposomes having 50 mol% cholesterol, but less than 4% glucose release from liposomes having 43 mol% cholesterol. Thus, even at our lowest levels, compared to the amounts used by Duncan and Schlegel, we were using more than 3000 times as much toxin, based on hemolytic units. Maximal glucose release under optimum conditions (at 50 mol% cholesterol) occurred at a toxin/phospholipid ratio of approx. 810 000 hemolytic units/μmol, or more than 20 000 times the amount employed by Duncan and Schlegel [5]. Cowell and Bernheimer [7] used about 10 000 hemolytic units of cereolysin/μmol phospholipid (Cowell, J.L., personal communication).

Cholesterol serves as a receptor for prymnesin, a toxin from alga [11], and for polyene antibiotics [12]. Incubation of liposomes containing cholesterol with prymnesin [11] or polyenes [13] results in glucose release. The polyenes also cause the appearance of liposomal rings that are similar to those caused by oxygen labile lysins [12]. The previously reported apparent absence of membrane damage to liposomes incubated with streptolysin O [5] has been cited as evidence that the lysin-induced rings seen in liposomes containing cholesterol may not be related to membrane damage or pore formation, and that the mechanism of lysin damage may be fundamentally different from that of polyene antibiotics [6]. Our data demonstrates that cholesterol-dependent

lysin-mediated liposomal membrane damage does occur.

The occurrence of maximal glucose release from liposomes having a phospholipid/cholesterol ratio of 1.0 (Fig. 1) suggests that the phospholipid membrane may play an important role in tetanolysin-mediated membrane damage. A popular theory of the mechanism of permeability caused by oxygen-labile toxins is that the cholesterol moves laterally in the membrane to complex with the toxin, and that the membrane becomes unstable because of the elimination of the interaction of cholesterol with phospholipids [1,9]. Liposomes comprised of egg phosphatidylcholine or dimyristoyl phosphatidylcholine are unstable (i.e. release most of their trapped glucose) at 24°C in the absence of cholesterol, but liposomes containing dipalmitoyl, or distearoyl phosphatidylcholine are not unstable at 24°C [14]. We found that liposomes containing dimyristoyl, dipalmitoyl, or distearoyl phosphatidylcholine were equally sensitive to lysin-induced damage at 24°C (Fig. 3) and our findings therefore do not fully support the above hypothesis [1,9]. These data are consistent with the concept that a toxin-related pore occurs in the phospholipid membrane, and that the pore may consist of a complex of toxin and cholesterol.

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