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Structural Characteristics of Tetanolysin and Its Binding to Lipid Vesicles

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Tetanolysin binding to lipid vesicles was found to depend on the molar ratio of cholesterol to phospholipid, being low in vesicles containing up to 20 mol% cholesterol and high in vesicles containing more than 33 mol%. High concentrations of purified tetanolysin preparations formed arc- and ring-shaped structures. The structures were not readily detectable in diluted preparations unless incubated with lipid vesicles containing high molar ratios of cholesterol to phospholipid. It is suggested that the toxin is concentrated on the vesicles to local concentrations high enough to form the arcs and rings.

Tetanolysin is a cytolytic toxin which interacts with biological as well as with artificial membranes (1, 4, 7, 9, 14), causing permeability changes (1, 4) and subsequent cell lysis (4, 14). Like other oxygen-labile bacterial hemolysins (2, 5), tetanolysin has cholesterol as its membrane receptor (12, 14). Recently, it was found that permeability changes induced by tetanolysin are most pronounced in lipid vesicles containing a high molar ratio of cholesterol to phospholipid (1). Therefore, the possibility that the ratio of cholesterol to phospholipid in lipid vesicles is crucial in determining the tetanolysin binding capacity of lipid vesicles is of great interest.

Our studies show that the extent of tetanolysin binding to dimyristoylphosphatidylcholine (DMPC)-cholesterol vesicles depends on both the concentration of cholesterol and the molar ratio of cholesterol to phospholipid (Fig. 1 and 2). Cholesterol was not required to prevent the crystallization of the saturated acyl chains of DMPC since at the experimental temperature (37°C) DMPC has fluid properties, being well above its phase transition temperature (24°C). Thus, our results support previous studies indicating a direct interaction of tetanolysin with membrane cholesterol (1, 4, 14). The binding of tetanolysin to DMPC vesicles containing up to 20 mol% of cholesterol was low. The binding capacity increased above 20 mol%, with maximal binding obtained with vesicles containing more than 33 mol% cholesterol (Fig. 1). It was possible to vary the molar ratio of cholesterol to phospholipid while maintaining a constant concentration of cholesterol in the reaction mixture

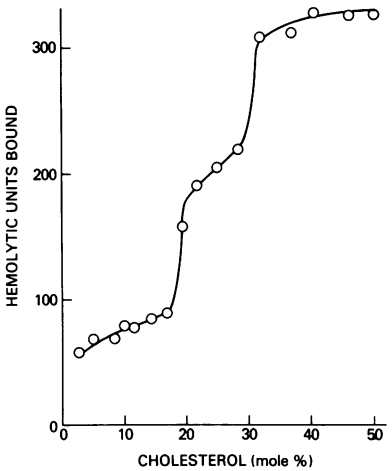


FIG. 1. Binding of tetanolysin to DMPC-cholesterol vesicles of different molar ratios. Lipid vesicles were prepared from L-α-DMPC and cholesterol at various molar ratios as previously described (15). The lipids were dispersed in phosphate-buffered saline (pH 7.4) by vigorous mixing for 2 min with a Vortex mixer. To 0.1-ml volumes of lipid vesicle suspensions containing cholesterol, 0.1 ml of a 1:10 dilution of the tetanolysin preparation was added. The mixtures were incubated for 15 min at 37°C, and the resulting lipid-tetanolysin complex was sedimented by centrifugation in an Eppendorf Micro Centrifuge at 12,800 × *g* for 2 min. Under these conditions, 99% of the lipid sedimented to the bottom of the tubes. The amount of tetanolysin bound to the lipid dispersions was calculated indirectly by determining the residual free tetanolysin activity in the supernatant fluid by following the hemolytic activity of the preparation (14). Hemolytic activities were expressed as HU based on the reciprocal of toxin dilution that caused 50% hemolysis (14).

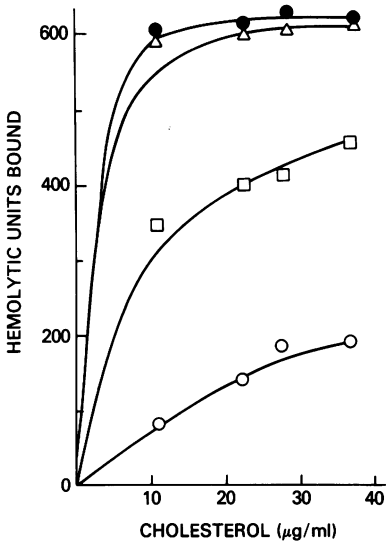


FIG. 2. Effect of cholesterol concentration on the binding of tetanolysin to lipid vesicles. The preparation of the vesicles and the binding experiments were performed as described in the legend of Fig. 1. Cholesterol concentration in the reaction mixture was changed by adding various amounts of DMPC-cholesterol vesicles containing different mole percentages cholesterol. Symbols: ○, 11 mol%; □, 22 mol%; △, 33 mol%; ●, 50 mol%.

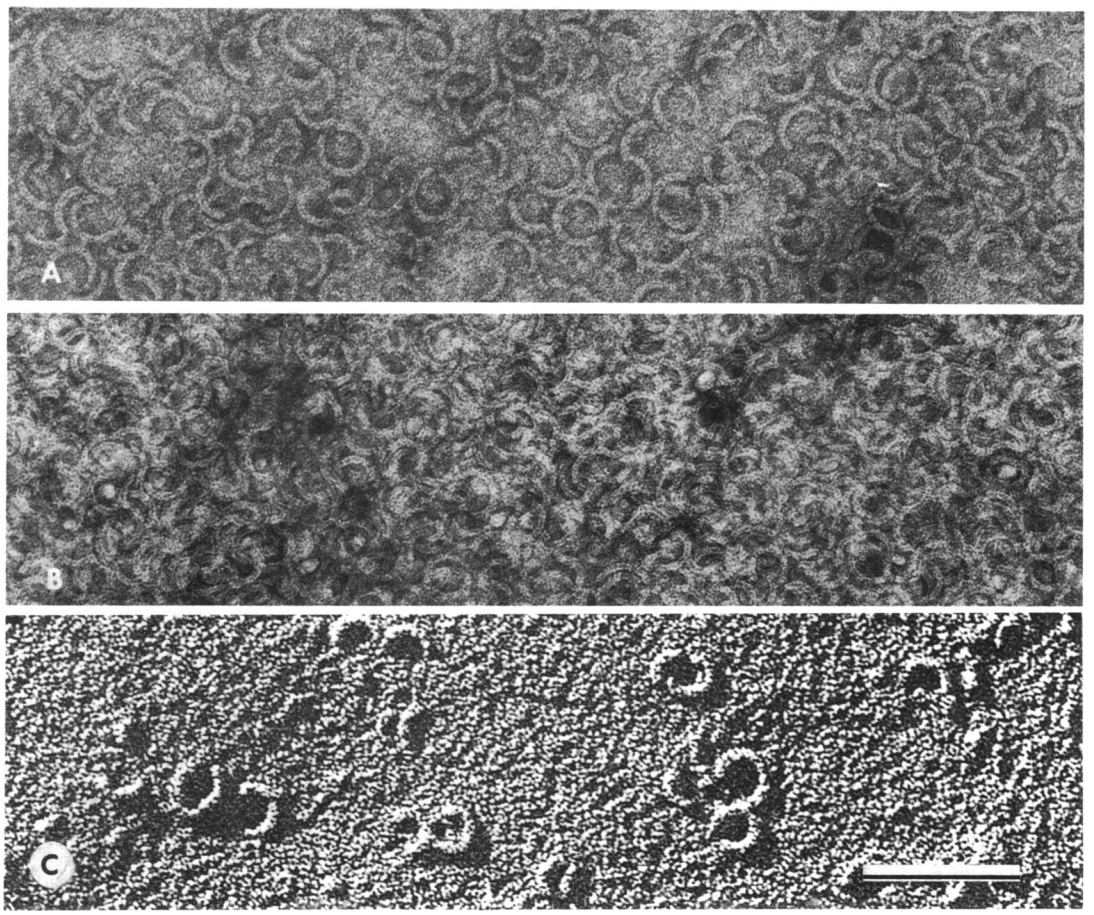


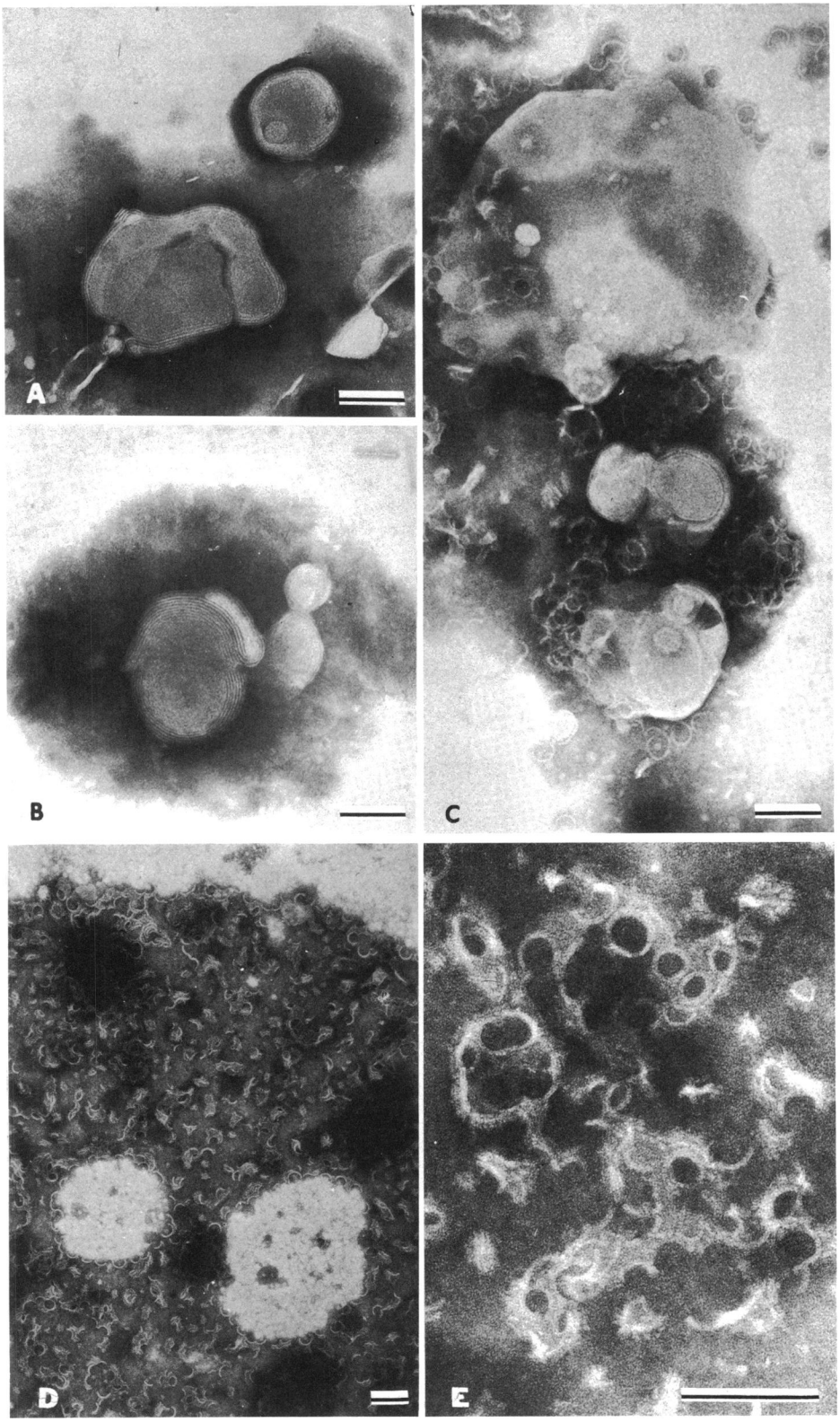
FIG. 3. Purified tetanolysin as seen by transmission electron microscopy. Tetanolysin was prepared as previously described (1, 8) and contained 25,600 HU/ml. Drops of tetanolysin in phosphate-buffered saline were placed on Formvar-coated grids. After 1 to 2 min, excess fluid was withdrawn by filter paper, and the grids were immediately negatively stained with either 2% potassium phosphotungstate (A) or 1% uranyl acetate (B). Duplicate samples, first fixed by a 20-min exposure to vapor from 50% glutaraldehyde, were similarly treated and stained. Some grids containing tetanolysin were allowed to dry after the removal of excess fluids and were subsequently shadowed with platinum-palladium at an angle of 20° in a Mikros Vacuum Evaporator VE 10. Samples of tetanolysin had their excess fluid absorbed onto the surface of 3% Noble agar and were replicated with 3% Parlodion. The replicas were floated off on distilled water, picked up on grids, and similarly shadowed (C). All grids were examined and photographed with a model HU11C Hitachi electron microscope operated at an accelerating voltage of 75 kV. Bar, 100 nm.

FIG. 4. Electron micrographs of interactions of purified tetanolysin (2,560 HU/ml) with DMPC-cholesterol vesicles. The preparations were stained with 3% phosphotungstic acid as described in the legend to Fig. 3. The vesicles were prepared as described in the legend to Fig. 1. (A and B) Dispersions with no cholesterol; (C) dispersions with 20 mol% cholesterol; (D and E) dispersions with 50 mol% cholesterol. (D) shows the center and edge (top of photo) of a large fragmented liposome with arcs and rings. (E) is a higher magnification of the same. All bars, 100 nm.

by adding different amounts of lipids to the reaction mixture. At any cholesterol concentration, tetanolysin binding to vesicles of 33 or 50 mol% cholesterol was markedly higher than binding to vesicles containing 22 mol% cholesterol, and the binding to lipid vesicles containing 11 mol% was very low (Fig. 2). These results indicate that the binding capacity of lipid vesicles is due mainly to differences in the molar ratio of cholesterol to phospholipid rather than to differences in the absolute cholesterol concentration. Ample evidence exists in the literature for changes in the properties of dipalmitoylphosphatidylcholine (DPPC)-cholesterol vesicles in the neighborhood of 20 mol% cholesterol (7, 16, 17), and some investigations have suggested that 33 mol% is a crucial concentration as well (6, 10). Such changes were shown to exist in lecithins other than DPPC, such as DMPC and egg lecithin, although the latter contains a mixture of saturated and unsaturated fatty acyl chains (16). It is, therefore, suggested that the changes occurring in the properties of the membrane bilayer at about 20 and 33 mol% cholesterol are accompanied by changes in the binding of tetanolysin. The changes in binding characteristics may explain previous observations that the lytic effects of tetanolysin (1) as well as streptolysin O (13) and cereolysin (2) depend on the ratio of cholesterol to phospholipid, being high at about 50 mol% but low or even nonexistent below 33 mol%.

Our electron microscopy studies show that purified tetanolysin in adequate concentrations (25,000 hemolytic units [HU]/ml) can, like cereolysin (3) and perfringolysin O (11), be visualized as arc- and ring-shaped structures by negative staining electron microscopy as well as in shadowed preparations and replicas (Fig. 3). Fixation by glutaraldehyde vapor, or omission of gelatin from the buffer, did not alter their presence or appearance. Only rare arcs could be found after a 1:10 dilution of the original tetanolysin preparation. The arcs consisted of linked subunits that appeared to retain stain in their centers. Arc length varied with the number of subunits from approximately 7 nm in short arcs to 18 to 20 nm in longer arcs and 30 nm in rings. The widths of the subunits were from 4 to 8 nm, whereas the internal diameters of the half-rings or rings were between 25 and 30 nm. The concentration dependency noted also by Cowell et al. (3) for cereolysin suggests that a critical concentration is required for the polymerization of hemolysin into arcs and that this concentration is produced on the grid by local conditions of drying or by negative stains if the concentration applied is in itself adequate. Possible structural differences between activated and nonactivated toxins, such as those described for perfringolysin O (11), remain to be examined. However, the dimensions of arcs and rings of tetanolysin are similar to those of cereolysin (thickness, 6.7 to 8.3 nm; outside diameter 33 to 50 nm) and perfringolysin O (thickness, 4 to 7 nm; inside diameter, 20 to 30 nm.

Rings and arcs were not readily detectable in diluted tetanolysin solutions (2560 HU/ml); however, when mixed with lipid vesicles these structures appeared around, bound to, or reacting with lipid vesicles in numbers that increased with increasing ratios of cholesterol to phospholipid. Thus, without cholesterol the multilamellar lipid vesicles were intact (Fig. 4A and B), occasionally, a few arcs could be seen in the surrounding stain. At a cholesterol content of 20 mol%, some vesicles were intact, and others were fragmented; rings and arcs were seen in, on, or around the lipid vesicles (Fig. 4C). The appearance was similar at 30 mol% cholesterol, but fewer intact and more fragmented vesicles were seen. At cholesterol contents of 40 and 50 mol%, the majority of lipid vesicles were converted to vesicle-shaped and vesicle-sized masses of rings and arcs and residual lipid vesicle fragments (Fig. 4D and E). These findings confirm the results of the binding experiments, which show that the affinity of tetanolysin for lipid vesicles is dependent on the ratio of cholesterol to phospholipid. It seems possible that this toxin, because of an affinity for cholesterol, is concentrated on or around the lipid vesicles, and the local high concentration may lead to polymerization into arcs and rings. The greater concentration on lipid vesicles with higher cholesterol content ($\geq$33 mol%) may account for the larger numbers of rings seen on the surface of the lipid vesicles as compared with their relative rarity in the preparations of purified toxin alone. It is not clear what role, if any, these structures play in the fragmentation of cholesterol-containing membranes.


A
B
C
D
E

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