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ALTERATION BY CEREOLYSIN OF THE STRUCTURE OF CHOLESTEROL-CONTAINING MEMBRANES

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Summary

When erythrocyte membranes were treated with cereolysin, negatively stained and examined by electron microscopy, ring and arc-shaped structures were observed in the membrane. The outside diameter of the rings varied from 33 to 50 nm with a border thickness of 6.7 to 8.3 nm. The arcs varied in length from 33 to 170 nm with a border thickness of also 6.7 to 8.3 nm. When right-side-out erythrocyte ghosts which had been treated with cereolysin were examined by electron microscopy after freeze-fracture, structures with a diameter of 31 to 63 nm were seen in the fracture face of the exoplasmic half of the membrane, but no alterations were visible in the fracture face of the protoplasmic half of the membrane bilayer. Thus the ring structures did not appear to form holes through the membrane. At cereolysin concentrations above 6 μg/ml rings and arcs were seen when purified toxin alone was examined. At or below 6 μg/ml toxin rings and arcs were seen only if toxin was incubated with free or membrane-bound cholesterol. Our interpretation is that cereolysin tends to aggregate into ring and arc-shaped structures, and that the tendency to aggregate is increased by cholesterol. Rings and arcs were not seen when erythrocyte ghosts were treated with low, but lytic amounts of cereolysin that significantly altered the permeability of the ghosts.

Introduction

Cereolysin is a bacterial toxin secreted by a variety of strains of *Bacillus cereus* [1]. The toxin has been purified to homogeneity [2] and shown [1--4] to have properties in common with the thiol-activated cytolytic toxins. The most thoroughly studied toxins of this group are cereolysin, streptolysin O,

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pneumolysin, perfringolysin O and tetanolysin. These toxins have many similar structural and biological properties [5—8], and it has been proposed that they have a common mode of action in altering membrane structure [5,8]. There is convincing indirect evidence, reviewed by Bernheimer [5] and Freer and Arbuthnott [7], that cholesterol is the membrane binding site for thiol-activated toxins. However, it is still not known how these toxins produce the membrane changes which result in lysis or death of sensitive cells.

In 1966 Dourmashkin and Rosse [9] reported that ring structures were seen in electron micrographs of negatively stained membranes that had been treated with streptolysin O. They suggested that these rings were holes in the membrane. In 1974 Bernheimer [5] proposed that lysis of cells by streptolysin O may be due to the escape of molecules through these holes which could be lined by cholesterol · streptolysin O complexes. Besides streptolysin O [9,10] similar, or identical, ring structures have been seen in membranes treated with perfringolysin O [11] and cereolysin [12]. Streptolysin O [10] and perfringolysin O [11] have been shown to form the ring structures, along with arc-shaped structures, after interaction with cholesterol. Despite these findings there was no evidence to show that these structures actually produced functional pores in membranes, and it is not clear what role the ring and arcs have in the lytic mechanism of thiol-activated toxins.

In this communication we show that cereolysin will also form ring- and arc-shaped structures by interaction with cholesterol or cholesterol-containing membranes. Using freeze-fracture electron microscopy it appears that these structures do not form holes through the membrane. We also suggest that these structures may be polymerized forms of cereolysin.

Materials and Methods

Cereolysin preparation

Cereolysin was assayed and purified as described [2], except the secondary culture supernatant was precipitated with 55% $(NH_4)_2SO_4$ and isoelectric focusing in a pH gradient of 3.5—10 was followed by focusing in a pH gradient of 6—8. Also, the Sephadex G-100 column chromatography was not always needed to obtain a pure protein. The toxin appeared homogeneous as previously shown [2], and its specific acitivity ranged from $4 \cdot 10^6$ to $5 \cdot 10^6$ hemolytic units/mg of protein.

Preparation of erythrocyte ghost membranes

Right-side-out and sealed erythrocyte ghosts were prepared by the methods of Steck [13] from human blood-bank blood, which was 3—4 weeks old. Cells were washed with 150 mM NaCl/5 mM sodium phosphate (pH 8.0), and ghosts were formed by osmotic lysis by dilution of the cells into cold 5 mM sodium phosphate (pH 8.0). The ghosts were resealed by incubation for 40—60 min at 37°C in 150 mM NaCl/5 mM sodium phosphate, pH 8.0. The sidedness and sealing of the ghosts were assayed using the accessibility of acetylcholinesterase and glyceraldehyde-3-phosphate dehydrogenase as described [13].

Preparation of bacterial cells and membranes

Total membrane fractions were prepared from *Micrococcus lysodeikticus* as described [14] and stored at −20° C at a concentration of 2.3 mg protein/ml.

Strain A of *Acholeplasma laidlawii* was grown in 100 ml PPLO broth (Difco Laboratories), pH 7.8, containing 0.5% (w/v) glucose and 1.0% (w/v) yeast extract (Difco Laboratories). Growth was for 12—21 h in 500-ml flasks incubated statically at 37° C. The cells incorporated cholesterol (3.7—7.2 μg cholesterol/mg cell protein) when the medium was supplemented with 20% (v/v) normal horse serum (New York City Department of Health). Cells containing no detectable cholesterol were obtained by replacing the horse serum with 0.5% (w/v) bovine serum albumin low in fatty acids (Sigma Chemical Co.). The cells were harvested by centrifugation and washed once with 0.25 M NaCl.

Protein and cholesterol determinations

Protein was assayed by the method of Lowry et al. [15], with bovine serum albumin (Sigma Chemical Co.) as the standard. After extraction of lipids from membranes using 2 : 1 (v/v) chloroform/methanol as described by Albrink [16], the lipids were saponified and total cholesterol determined as described by Rudel and Morris [17].

Preparation of lipid dispersions

Liposomes composed of phosphatidylcholine (10 μmol)/cholesterol (10 μmol)/dicetylphosphate (1 μmol) or phosphatidylcholine (10 μmol)/dicetylphosphate (1 μmol) were prepared by mixing the various lipids, dissolved in chloroform/methanol (1 : 1, v/v), and drying them under vacuum or nitrogen. The lipids were resuspended in 77 mM NaCl/67 mM sodium phosphate buffer (pH 7.0) and dispersed by sonicating for 90 s in a sonic water bath (Millipore Corporation). The suspensions were then adjusted to an absorbancy of 0.8 at 650 nm using a 1-cm cell in a Beckman DU spectrophotometer. Dispersions of cholesterol (29 mg/ml) alone were similarly prepared.

Electron microscopy

For negative staining, a drop of the appropriate suspension or solution was placed on a Formvar, carbon-coated grid for 1 or 2 min and the excess fluid removed with a small piece of filter paper. The grids were stained immediately with 2% ammonium molybdate. In some experiments the samples were stained with 1.0% phosphotungstic acid or 0.1% uranyl acetate. Cereolysin was diluted with 77 mM NaCl/67 mM sodium phosphate buffer, pH 7.0, containing 0.5 mM dithiothreitol and 0.1% (w/v) gelatin. In some experiments the gelatin was either omitted or replaced by 0.005—0.05% bovine serum albumin.

For freeze-fracturing, samples were suspended in 30% glycerol for 30 min at 26° C before centrifugation. A small portion of the pellet was frozen in Freon 22. Freeze-fracturing was done in a Balzer Freeze-etch apparatus as described [18]. The replicas were cleaned in commercial sodium hypochlorite, rinsed in demineralized water and mounted directly on uncoated copper grids. Specimens were examined in a Siemens Elmiskop I equipped with a decontamination device.

Ultracentrifugation

Sedimentation velocity and equilibrium experiments were done with a Beckman-Spinco model E analytical ultracentrifuge equipped with an ultraviolet absorption scanner. These analyses were done with a protein concentration of 0.24 mg/ml. Cereolysin was in 0.05 M sodium phosphate buffer, pH 7.0, for sedimentation velocity and in the same buffer containing 0.1 mM dithiothreitol and 6% glycerol for sedimentation equilibrium. For calculations the partial specific volume of cereolysin was assumed to be 0.74 ml/g. The density of the buffer used in sedimentation equilibrium was measured using a 100 ml volumetric flask and calculated to be 1.027 g/ml at 10° C. Other necessary density and viscosity values were obtained from appropriate tables. Sedimentation velocity was done at 40 000 rev./min at 5° C. Sedimentation equilibrium was done at 10° C (18 000 rev./min) and at 5° C (6400 rev./min). Equilibrium was established after 22 h.

Chemicals

Synthetic lecithin (DL-α-phosphatidylcholine dipalmitoyl), cholesterol and dicetylphosphate were obtained from Sigma Chemical Co., St. Louis, Missouri. 5,5′-dithiobis (2-nitrobenzoic acid) was from Nutritional Biochemicals Corp. All other chemicals were reagent grade and obtained from usual commercial sources.

Results

Ring and arc formation by interaction of cereolysin with membranes

When erythrocytes or erythrocyte ghost membranes were treated with cereolysin at 5° C or 37° C, ring- and arc-shaped structures were visible in the membranes by electron microscopy after negative staining (Fig. 1). Identical structures were seen when cereolysin was incubated with phosphatidylcholine-cholesterol liposomes and aqueous dispersion of cholesterol. The outside diameter of the rings varied from 33 to 50 nm with a border thickness of 6.7—8.3 nm. The arcs varied in length from 33 to 170 nm with a border thickness of also 6.7—8.3 nm. After interaction with free or membrane-bound cholesterol, the lowest concentration of toxin showing rings and arcs was 0.5 μg/ml. At this concentration very few rings and arcs were visible, but their number increased with increasing amounts of toxin. Cereolysin at or below 6 μg/ml did not reveal rings and arcs until treated with free or membrane-bound cholesterol; the erythrocyte membranes, aqueous dispersions of cholesterol, or liposomes containing cholesterol did not show rings and arcs until treated with cereolysin.

It appears that cholesterol can induce ring and arc formation since only cholesterol or cholesterol-containing membranes form these structures after interaction with cereolysin. Liposomes lacking cholesterol or membranes (1.2 mg protein/ml) from the bacterium *M. lysodeikticus* did not form rings and arcs when treated with cereolysin (6 μg/ml; 28 000 hemolytic units/ml) for 30 min at 37° C. Also, after similar treatment with 21.3 μg/ml and 62 000 hemolytic units/ml of cereolysin, *A. laidlawii* grown in the presence of cholesterol formed rings and arcs in the membranes, but *A. laidlawii* grown in the absence of cholesterol did not.

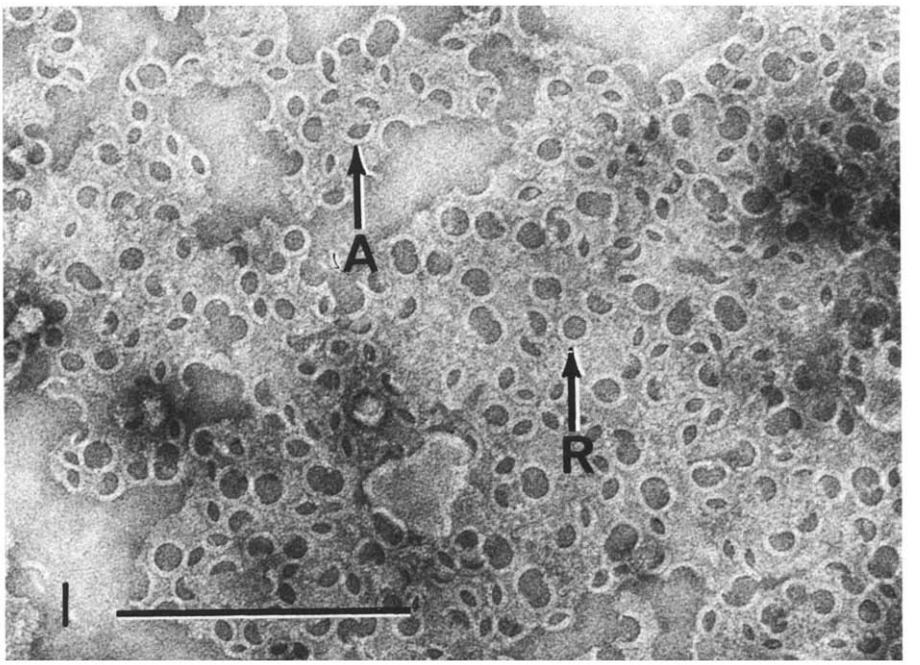

Fig. 1. Human erythrocytes treated with cereolysin. Erythrocytes (0.7%, v/v, suspension) were treated with toxin (14 μg/ml; 40 000 hemolytic units/ml) at 37°C for 15 min. Membranes were obtained by centrifugation at 27 000 × g for 20 min and negatively stained. Magnification, ×78 000; bar = 500 nm. Rings, arrow R; arcs, arrow A.

Ring and arc formation with purified cereolysin alone

In the absence of added membranes or cholesterol, purified cereolysin at concentrations greater then 6 μg protein/ml also showed ring and arc-shaped structures by electron microscopy after negative staining (Fig. 2) or shadow casting. The ring and arc structures seen with the toxin alone appeared identical to those formed in the presence of cholesterol, and they also increased in number with increasing concentrations of cereolysin. The microscopic field was very dense with these structures at toxin concentrations of 110—240 μg protein/ml.

Since the rings and arcs might be polymerized forms of cereolysin, it seemed possible that they could be separated, on the basis of size, from non-polymerized cereolysin, which was previously shown to have a molecular weight of about 55 500 [2]. However, subjecting cereolysin at a concentration of 240 μg/ml to sedimentation velocity and sedimentation equilibrium as described in Materials and Methods did not reveal large molecular weight components. Cereolysin behaved as a homogeneous protein with a sedimentation coefficient of 3.2 S and a molecular weight of about 50 000. Also, 420 μg of purified toxin were applied to a Sephadex G-100 column (1.0 × 24 cm) which was operated as previously described [2]. Cereolysin eluted from this column as a single protein peak which was coincident with a single hemolytic peak and again had a molecular weight of about 50 000. Electron microscopy revealed many ring

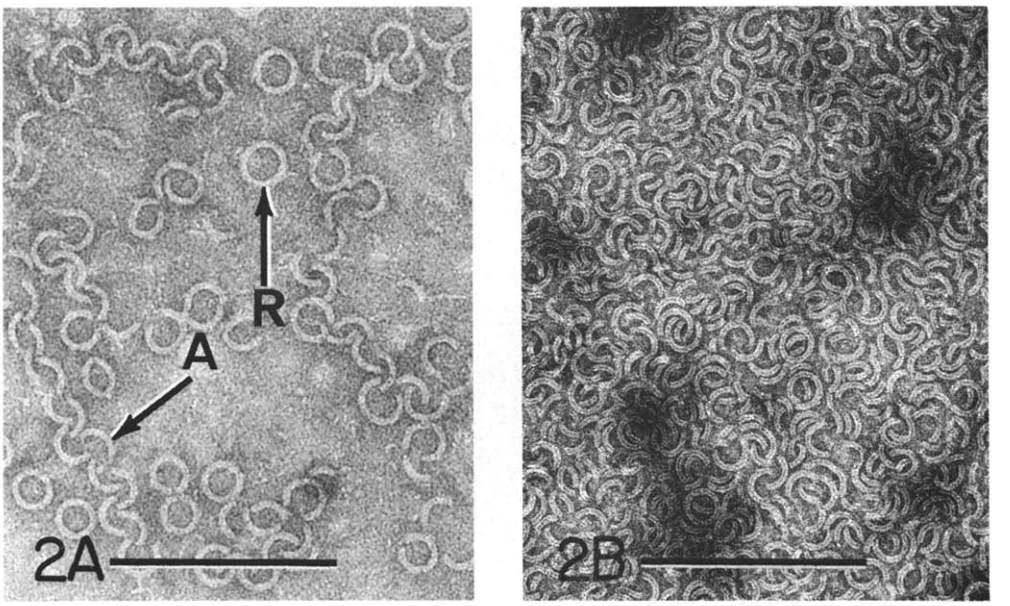

Fig. 2. Rings (arrow, R) and arcs (arrow, A) seen in purified cereolysin by negative staining. 2A, Toxin at 110 μg/ml; 2B, toxin at 240 μg/ml. Magnification: ×120 000; bar = 250 nm.

and arc structures in the fractions comprising this protein peak. No detectable protein was eluted at the void volume of the column, and no rings and arcs were detectable in those fractions that contained the void volume of the column.

Freeze-fracture electron microscopy

The erythrocyte ghost membrane appeared as reported elsewhere [19] when viewed by electron microscopy after freeze-fracture. The PF face (fracture face of the protoplasmic half) contained large numbers of randomly distributed intramembranous particles, while the EF face (fracture face of the exoplasmic half) contained only a small number of particles. When these erythrocyte ghosts were treated with cereolysin a significant alteration occurred in the structure of the membrane as revealed by freeze-fracture electron microscopy (Fig. 3). Structures with a diameter of 31—63 nm were seen on the EF face of the membrane. Although these structures appeared to protrude toward the cytoplasmic side of the outer half of the membrane, no alterations were seen in the PF face of the membrane.

Relationship between ring and arc formation and lytic activity

To determine whether the ring and arc structures were essential to the lytic mechanism of cereolysin, erythrocyte ghosts, which were 97% sealed and right-side out, were incubated with increasing concentrations of cereolysin, and the ability to see ring and arc structures in the membranes was followed along with the ability of the toxin to alter the permeability of the ghosts. The results are shown in Table I. Changes in the permeability of the membrane of the ghosts

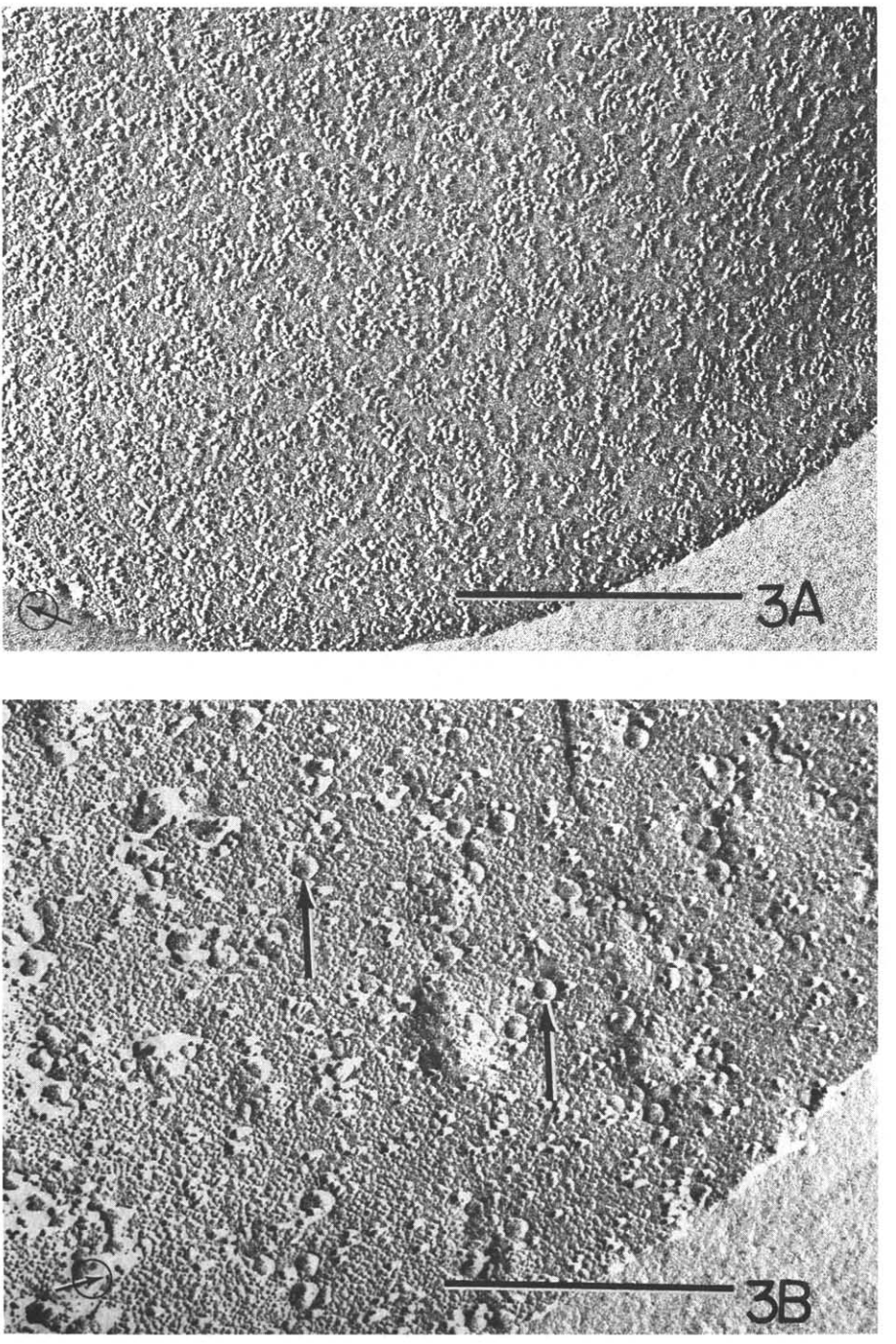

Fig. 3. Effect of cereolysin on human erythrocyte ghosts as seen by freeze-fracture electron microscopy. Erythrocyte ghosts (3.5 mg membrane protein/ml) were treated with toxin (90 µg/ml) for 15 min at 37°C. Circled arrows indicate direction of shadowing. Magnification, ×78 000; bar = 500 nm. A, PF face of membrane shows no alteration in structure from control membranes. B, EF face reveals alterations in the form of structures (arrows) with a diameter of 31—63 nm among the normal small number of randomly distributed intramembranous particles.

TABLE I

INTERACTION OF CEREOLYSIN WITH SEALED, RIGHT-SIDE-OUT ERYTHROCYTE GHOSTS

Erythrocyte ghosts which were 97% sealed and right-side-out were incubated at 0.9 mg of membrane protein/ml for 30 min at 37°C with cereolysin. Alteration of the permeability of the ghosts was estimated by assaying glyceraldehyde-3-phosphate dehydrogenase activity as decribed in Materials and Methods. The activity of the enzyme after treating the ghosts with 0.05% sodium dodecyl sulfate was 1.8 μmol/min per mg protein and was taken as 100% permeability. Ring and arc formation was determined by electron microscopy after negative staining.

Cereolysin		Sealed ghosts (% permeability)	Rings and arcs
(Hemolytic units/ml)	(μg/ml)		
150	0.036	33	None
375	0.090	54	None
750	0.18	75	None
1500	0.36	88	None
75 *	1.8	20	Yes
150 *	3.6	40	Yes

* Before incubation with ghosts, cereolysin was partially inhibited by incubation with 2 mM 5,5'-dithiobis (2-nitrobenzoic acid) for 20 min at 37°C.

were monitored by the ability of glyceraldehyde-3-phosphate to reach glyceraldehyde-3-phosphate dehydrogenase which is present on the cytoplasmic surface of the erythrocyte ghost membrane. At cereolysin concentrations that resulted in the expression of up to 88% of the activity of this enzyme no rings and arcs could be seen in the membranes by electron microscopy after negative staining. Repeated experiments gave similar results.

It is also shown in Table I that if the activity of cereolysin was inhibited 99% by incubation with 5,5'-dithiobis (2-nitrobenzoic acid), erythrocyte ghosts could be exposed to 1.8 and 3.6 μg toxin/ml with only a 20—40% change in the permeability of the ghosts, respectively. Under these conditions ring and arc structures could be seen in the membranes. When the results in Table I are compared it appears that the ability to see rings and arcs correlates better with the protein concentration of cereolysin than with the lytic activity of the toxin. At a concentration of 3.6 μg/ml, cereolysin which was inhibited by the above sulfhydryl reagent appeared to form about the same number of rings and arcs in erythrocyte ghost membranes as highly active toxin at the same protein concentration.

Different results were obtained when small amounts of cereolysin were incubated with intact erythrocytes. Incubation of 1 ml of a 0.7% (v/v) suspension of human or rabbit erythrocytes with cereolysin at 5 hemolytic units/ml and 1.8 ng of protein/ml resulted in 90% lysis of the cell suspension after 30 min at 37°C. In the membrane of these lysed cells were seen hole-like alterations irregular in shape and size (Fig. 4). Similar incubation of the erythrocytes with cereolysin at 21 and 210 hemolytic units/ml resulted in complete lysis of the cells at 37°C within 5 and 1 min, respectively. The structures shown in Fig. 4 became more difficult to find in the membranes of the lysed cells as the toxin concentration was increased. Definite ring and arc-shaped structures were seen only when erythrocytes were lysed by cereolysin at or above 0.5 μg of toxin/ml. It is difficult to be certain of the relationship of the alterations shown in

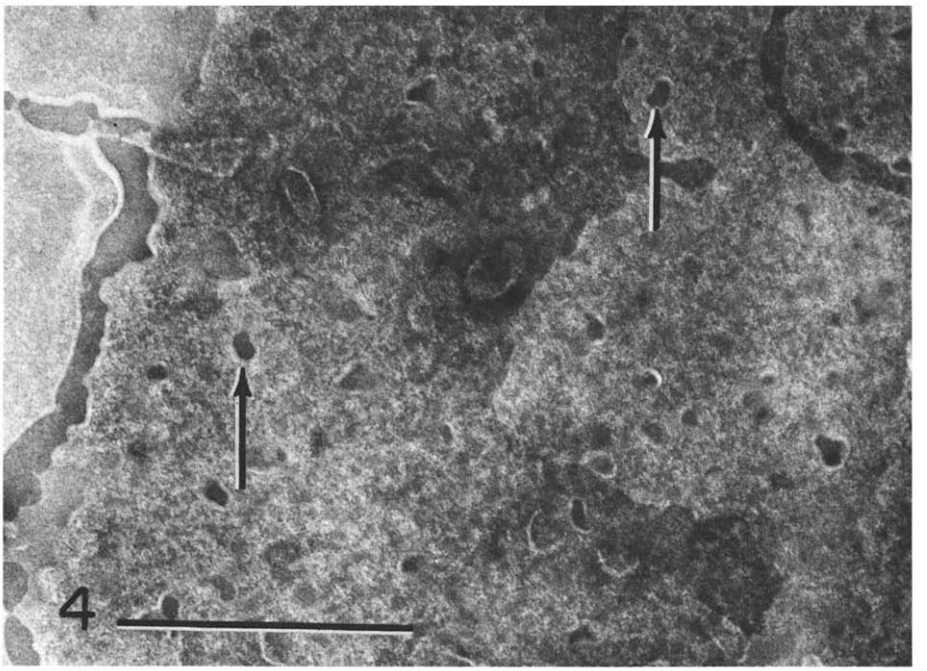

Fig. 4. Negatively-stained erythrocyte membranes treated with cereolysin. Intact human erythrocytes (0.7%, v/v, suspension) were lysed by cereolysin (1.8 ng/ml; 5 hemolytic units/ml) at 37°C for 30 min. Membranes were obtained by centrifugation. Magnification, ×78 000; bar = 500 nm. Arrows show alterations caused by cereolysin.

Fig. 4 to either the lytic mechanism of cereolysin or to the ring and arc-shaped structures.

Results with *A. laidlawii* are also relevant to the relationship of the ring and arcs to lysis. When *A. laidlawii* cells (0.89 mg protein/ml) which contained cholesterol in their membranes were incubated with cereolysin (21.3 μg/ml; 62 000 hemolytic units/ml) for 30 min at 37°C rings and arcs could be seen in the membranes. However, there was little or no lysis of the cells as evidenced by only a 7% decrease in the absorbancy of the cell suspension which had an absorbancy of 0.6 at 500 nm without the addition of toxin.

Discussion

Our results show that cereolysin forms ring and arc-shaped structures very similar, if not identical, to those formed by other thiol-activated toxins [10, 11]. With streptolysin O [10] these structures were not observed unless the toxin was incubated with cholesterol or cholesterol-containing membranes, and it was suggested that these rings and arcs might be aggregates of a streptolysin O · cholesterol complex, a repacking of cholesterol molecules alone, or unfolded streptolysin O in an α-helical form with cholesterol molecules attached. Duncan and Schlegel [10] formulated and favored the latter hypothetical model primarily because most of the arc-shaped structures had a length

of 75 nm. However, with cereolysin and with perfringolysin O [11] the lengths of the arcs vary considerably. Using perfringolysin O the rings and arcs could occasionally be seen with the purified toxin alone, but were seen in abundance when the toxin was incubated with cholesterol. Based on the finding of these structures in highly purified perfringolysin O, Smyth, Freer and Arbuthnott [11] suggested that the rings and arcs may be polymeric forms of toxin only. The formation of rings and arcs with cereolysin also appeared to be induced by the presence of cholesterol, but unlike the other toxins, the rings and arcs were seen in large numbers when the purified toxin alone was examined.

The finding of these structures in highly purified preparations of perfringolysin O and cereolysin suggests that the rings and arcs may indeed be polymeric forms of these toxins. Under yet undefined conditions, these toxins may aggregate and the ability of the toxins to aggregate may be enhanced by cholesterol. This could be analogous to the spontaneous polymerization, and more extensive polymerization induced by lipid, shown to occur with alpha toxin from *Staphylococcus aureus* [20]. However, unlike alpha toxin the polymerization of thiol-activated toxins is enhanced specifically by cholesterol. Thus, membranes lacking cholesterol do not form arcs and rings when treated with cereolysin. If the rings and arcs seen in purified cereolysin are a cereolysin · cholesterol complex then contaminating cholesterol must have been present, and the complex must have formed either in the original culture or during purification. We consider the polymerization theory more likely to be correct than the theory of toxin · cholesterol complex formation. Since the rings and arcs were not resolved from monomeric cereolysin by ultracentrifugation or G-100 column chromatography, and since they were seen in the purified toxin using three different negative stains and shadow casting, we offer the following explanation for the observation of rings and arcs in purified cereolysin. At sufficient concentration of toxin, drying of the toxin on the grid or some property of the grid itself may produce areas of relatively high concentrations of toxin leading to polymerization to ring- and arc-forms. Although other explanations are possible, we consider this the simplest one consistent with our results. It may be difficult, however, to prove that cereolysin can aggregate on the grid under the conditions used.

Other lytic agents known to produce ring structures in membranes are filipin [21—23], saponin [9,24] and immune lysis by antibody and complement [9,25]. There are some properties common to filipin, saponin and the thiol-activated toxins: only membranes that contain cholesterol are susceptible to lysis by these agents [5,26] and it is known that they will compete for a common binding site on the membrane [27]. The ring structures observed with the above lytic agents have been described as holes or pits in erythrocyte and artificial membranes. The question as to whether they extend through the full thickness of the membrane has been studied using freeze-fracture and freeze-etching. In the case of filipin [19,22,28], saponin [29] and immune lysis [25], the rings do not appear to be transverse holes in the membrane. Our freeze-fracture results with membranes treated with cereolysin indicate that the rings produced by cereolysin also do not transverse the membrane. The structural alterations produced by cereolysin were seen only in the outer half of the hydrophobic layer of the membrane and no changes were seen in the inner half of the mem-

brane bilayer. The structures seen by freeze-fracture in the EF face of membranes treated with filipin [19,28], and those produced by cereolysin appear to protrude toward the cytoplasm. However, filipin produces what appear as depressions or excavations in the PF face of the membrane [19], whereas cereolysin does not.

Data concerning the relationship of the rings and arcs to the lytic mechanism of cereolysin indicate that these structures may not be necessary for lysis. Evidence for this is: (i) at concentrations of cereolysin that caused extensive permeability changes in the membranes of erythrocyte ghosts no rings and arcs were seen by electron microscopy after negative staining. However, it may be that the number of rings and arcs present is too small to permit them to be seen. (ii) When highly active toxin was compared with cereolysin whose lytic activity was inhibited by a sulfhydryl reagent, the formation of rings and arcs appeared to correlate better with the protein concentration of the toxin than with its lytic activity. (iii) *A. laidlawii* cells, that contained cholesterol, formed rings and arcs in the membrane after treatment with cereolysin, but very little, if any, lysis of these cells occurred. (iv) Cereolysin and streptolysin O form rings and arcs in or on liposomes containing cholesterol, but neither cereolysin [30] nor streptolysin O [10,30] will cause the release of substances trapped in these liposomes.

A popular theory for lysis of cells by thiol-activated toxins [5,32] proposes that after the toxin binds to cholesterol in the membrane, the toxin · cholesterol complexes aggregate in the plane of the membrane and cause lysis because of increased membrane disorganization due to removal of cholesterol from its normal interaction with phospholipids. The formation by cereolysin of apparent aggregated areas in the hydrophobic core of the membrane is consistent with this theory. If the rings and arcs formed by interaction of thiol-activated toxins with cholesterol are eventually found to be composed of cholesterol and toxin molecules, this would obviously add support to the above theory. These structures have never been isolated or chemically defined with any of the lytic agents known to form them.

The above theory is similar to that outlined for lysis by filipin [28,31]. However, besides differing in chemical composition, filipin and thiol-activated toxins have other differences. Filipin causes lysis of *A. laidlawii* cells which contain cholesterol and also causes release of substances from cholesterol-containing liposomes. Data to date indicate that thiol-activated toxins do neither. This may suggest a fundamental difference in the mechanism of action of these lytic agents or may just be due to the ability of the smaller filipin molecules to gain access to and bind a larger percentage of the cholesterol in these particular membranes.

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