On the Mechanism of Membrane Damage by *Staphylococcus aureus* α-Toxin

ROSWITHA FÜSSLE, SUCHARIT BHAKDI, ANDREAS SZIEGOLEIT, JØRGEN TRANUM-JENSEN, THEODOR KRANZ, and HANS-JOBST WELLENSIEK
Institute of Medical Microbiology, D-6300 Giessen, Federal Republic of Germany, Anatomy Institute Department C, University of Copenhagen, The Panum Institute, DK-2200 Copenhagen N, Denmark, and The Behringwerke AG, D-3550 Marburg, Federal Republic of Germany

ABSTRACT Rabbit or human erythrocytes lysed with *Staphylococcus aureus* α-toxin were solubilized with Triton X-100, and the toxin was subsequently isolated by gel chromatography, sucrose density gradient centrifugation, and reincorporation into liposomes. In the presence of Triton X-100, the toxin exhibited a sedimentation coefficient of 11S and eluted at a position between those of IgG and α_2-macroglobulin in gel chromatography. A single polypeptide subunit of 34,000 mol wt was found in SDS PAGE. In the electron microscope, ring-shaped or cylindrical structures were observed, 8.5–10 nm in diameter, harboring central pits or channels 2–3 nm in diameter. An amphiphilic nature of these structures was evident from their capacity to bind lipid and detergent, aggregation in the absence of detergents, and low elutability from biological and artificial membranes through ionic manipulations. In contrast to the membrane-derived form of α-toxin, native toxin was a water-soluble, 34,000 mol wt, 3S molecule, devoid of an annular structure. Because studies on the release of radioactive markers from resealed erythrocyte ghosts indicated the presence of circumscribed lesions of ~3-nm effective diameter in toxin-treated membranes, the possibility is raised that native α-toxin oligomerizes on and in the membrane to form an amphiphilic annular complex that, through its partial embedment within the lipid bilayer, generates a discrete transmembrane channel.

Staphylococcus aureus α-toxin is a cytolytic exotoxin produced by most pathogenic staphylococci (1, 2, 6, 44). It is secreted as a water-soluble, 3S molecule whose molecular weight has been reported to be 28,000–40,000 (22, 41, 48, 55). The work of Arbuthnott et al. (4, 25, 26) early indicated that the primary target of α-toxin action is the lipid bilayer. Thus, the toxin binds to and damages artificial liposomal bilayers, causing release of low molecular weight markers (21, 25, 56). In induces an increase in electrical conductance of lipid monolayers (16). It lyses nucleated cells and erythrocytes, exhibiting a broad reactivity towards cells from different species (5, 7).

Binding of α-toxin to the membrane appears to be accompanied by an oligomerization of monomer, native 3S toxin to form a membrane-bound 12S complex (3, 25). Circumstantial evidence indicates that membrane-bound toxin hereby becomes intimately associated with the lipid bilayer (4, 25, 26). In electron micrographs, ring-shaped formations on toxin-treated membranes have been observed that are proposed to represent the membrane-bound 12S toxin oligomers (7, 25, 26).

The mechanism through which α-toxin damages membranes is, however, still unclear (40). The membrane-bound form of the toxin has not been isolated and little is known of its molecular nature. We have therefore sought to isolate α-toxin from target membranes, and to characterize the membrane-derived toxin with respect to its biochemical and ultrastructural properties, and to its capacity to generate functional membrane lesions. In this report, we present data indicating that α-toxin oligomerizes at the membrane surface to form amphiphilic, ring-shaped protein complexes. In conjunction with marker release data, the results are compatible with the concept that, analogous to the mechanism of immune cytolysis by complement (12, 39), toxin molecules self-associate to form ring structures that penetrate into or through the target lipid bilayer, generating transmembrane channels.

MATERIALS AND METHODS

Toxin

Staphylococcus aureus, strain Wood 46, was kindly supplied by Dr. H.-G.

Blobel (Institute of Bacteriology, Giessen). Stock preparations were cultured on sheep blood agar, and 100-ml batches of Tryptone Soya Broth (Difco Laboratories, Detroit, Mich.) were subsequently inoculated with bacteria taken from the surface of the agar. The bacteria were cultured in this medium on a rotary shaker (Finfors, Basel, Switzerland) at 37°C for 18 h. They were removed by centrifugation at 4°C (Sorvall centrifuge RC 2 B, rotor GSA, 10,000 rpm × 20 min; DuPont Co., Wilmington, Del.), and the resulting supernatant fluid was concentrated 10-fold by pressure dialysis (Amicon PM 10 membranes; Amicon Corp., Lexington, Mass.). The concentrated supernate exhibited a titer of ~2,048 in a hemolytic assay using standard commercial reagents (see below).

A preparation of purified α-toxin in lyophilized form was generously made available to us by the Behringwerke, Marburg, W. Germany. Immediately before use, 40 mg of the toxin preparation was dissolved in 10 mM Tris, 50 mM NaCl, 15 mM NaN₃, pH 8.2, and chromatographed in the same buffer over Sephacryl-S 300 (Pharmacia, Uppsala, Sweden; column dimensions: 2.6 cm diameter, 58 cm height) at 4°C. Two protein peaks were separated from each other. The first, accounting for ~25% of total protein and eluting in a molecular weight region of 180,000–500,000, was discarded. The second peak, eluting later at a K_{av} of 0.7, accounted for ~75% of total protein and represented the native α-toxin monomer. Fractions containing this protein peak were pooled and used in the experiments.

Treatment of Cells and Membranes with α-Toxin

Rabbit or human erythrocytes drawn in ACD (acid-citrate-dextrose) were washed three times and suspended in saline. 10% cell suspensions were lysed through addition of ⅓ vol of purified α-toxin (initial toxin concentration: 0.6–0.8 mg/ml; final toxin concentration: 0.15–0.2 mg/ml) and incubation at 37°C for 30 min. Equivalent results were obtained when hypotonically lysed, washed ghosts, rather than intact cells, were used.

When crude bacterial supernate was utilized as the toxin source, cells or washed membrane suspensions were similarly treated with 2–3 vol of 10-fold concentrated culture supernate, in the presence of 2 mM phenylmethylsulfonyl fluoride (PMSF). In all cases, the toxin-treated membranes were subsequently washed four times at 4°C with 5 mM phosphate, pH 8.0 (Sorvall centrifuge, RC 2 B, rotor SS 34; 16,000 rpm × 10 min).

Isolation of α-Toxin from Target Membranes

Toxin-treated membranes were solubilized in 1% (vol/vol) Triton X-100 (sinctillation grade, Serva). A clear detergent extract was obtained by subsequent centrifugation at 35,000 rpm (Beckman ultracentrifuge, rotor SW 50.1; Beckman Instruments, Inc., Palo Alto, Calif.) × 60 min, 4°C. α-Toxin was isolated from this detergent extract by three purification steps:

(a) Chromatography over Sephacryl S-300. This was performed on a 2.6 × 58 cm column equilibrated with 10 mM Tris, 50 mM NaCl, 15 mM NaN₃ (pH 8.2) containing 2.4 mM Triton X-100. 7–8 ml detergent extract were applied in each run, and 8-ml fractions were collected at a flow rate of 16 ml/h. The elution positions of blue dextran, $α_2$-macroglobulin, and IgG were determined. $α_2$-Macroglobulin and IgG were determined in whole human serum with the use of specific antibodies and fused rocket immunoelectrophoresis. All chromatography was performed at 5°C in a cold room.

(b) Sucrose density gradient ultracentrifugation. Fractions containing α-toxin recovered from the Sephacryl column were pooled, concentrated by pressure dialysis (Amicon PM 10 membranes) to ~3 ml, and 1-ml aliquots were applied to 10–43% (wt/wt) linear sucrose density gradients in the same buffer system, containing 1.2 mM Triton X-100. Centrifugation was performed in a Spinco ultracentrifuge (rotor SW 41 Ti; Beckman Instruments, Inc.) at 35,000 rpm for 16 h at 10°C. 20 equal fractions were collected from the bottom of the tubes. Sedimentation coefficients were approximated from a standard plot of fraction number (relative sedimentation distance) vs. sedimentation coefficient ($S_{20,w}$) of the following serum proteins: transferrin (5.5S), IgG (7S), C3 (9.5S), and IgM (19S) (38), using fused rocket immunoelectrophoresis (8).

(c) Reincorporation of α-toxin into artificial lipid vesicles. Fractions containing α-toxin recovered from sucrose density gradients were pooled and added to 3 vol of a stock α-lecithin solution (type V-E, 4 mg/ml; Sigma Chemical Co., St. Louis, Mo.) in 20 mM Tris, 50 mM sodium deoxycholate (DOC), and 15 mM NaN₃, pH 8.0. The samples were dialyzed against 400 vol of 20 mM Tris, 100 mM NaCl, 7.5 mM NaN₃ (pH 8.0) at 22°C for 48 h. Removal of detergent during this period of dialysis led to formation of lipid vesicles (liposomes).

The samples were subsequently concentrated to 1.5 ml over Amicon PM 10 membranes, supplemented with sucrose to a concentration of 50% (wt/wt), and overlayered with a 30% (wt/wt) sucrose solution in an ultracentrifugation tube. The lipid vesicles were flotated through sucrose solution by centrifugation at 40,000 rpm for 4 h at 15°C (rotor SW 50.1). Seven fractions were recovered from each flotation gradient.

Membrane Elution Experiments

The degree of elutability of α-toxin from biological and artificial membranes through treatment with various salt solutions was investigated in the following manner. For erythrocyte membranes, to 1 ml of packed membranes (in 5 mM phosphate, pH 8.0) was added 4.3 ml of one of the following buffers: (a) 1 mM EDTA, pH 8.0, (b) 10 mM EDTA, 1 M NaCl, pH 8.0, (c) 10 mM Tris, 5 mM p-chloromercuribenzenesulfonate (pCMBS; Sigma Chemical Co.) pH 8.0 (17), (d) 20 mM Tris, 1 M KCl, pH 8.0, or (e) 20 mM Tris, 1 M KI, pH 8.0. The samples were incubated at 37°C for 3–5 h and the membranes subsequently pelleted by centrifugation at 35,000 rpm × 60 min (Spinco ultracentrifuge, rotor SW 50.1). The membrane pellets were resuspended by addition of distilled water to the same volume as the elution supernates, and aliquots were applied to SDS gels and analyzed by rocket immunoelectrophoresis. For liposomes, reconstituted liposomes, harvested from the top of flotation gradients, were dialyzed against 100 vol of the same buffers stated above for 16 h at 22°C. Thereafter, reflotation of the liposomes was carried out through sucrose solutions. Fractions containing eluted protein from the bottom of the second flotation gradients, and those containing lipid-bound protein from the top of the gradients were analyzed by SDS gel electrophoresis and by immunoelectrophoresis.

Antisera and Antibodies

Antisera were raised in three rabbits by injection with the floated liposomes carrying α-toxin, following the immunization procedure of Harboe and Ingild (30). Antisera exhibiting satisfactory antibody titers were obtained after two booster injections. An antiserum pool was used to monitor the isolation of α-toxin from membrane detergent extracts (see Results).

Specific antibodies to α-toxin were isolated from the crude antiserum pool by a membrane elution procedure previously described (11). In brief, 2 vol of washed, toxin-treated membranes were mixed with 1 vol of antiserum. The adsorbed antibodies were subsequently eluted with 1 M acetic acid and purified by a single absoprtion/desorption step on protein A Sepharose. A purified immunoglobulin preparation containing ~0.8 mg/ml protein was radiolabeled with ¹²⁵I by the chloramine-T method (29). Radioactive antiglobulin tests performed basically as described (11) indicated a content of ~80% specific antibodies to α-toxin in this preparation. Briefly, radiolabeled antibodies (100,000 cpm) were incubated with toxin-treated erythrocyte membranes for 1 h at 20°C. After five washings in NaCl (Eppendorf centrifuge, 12,000 g × 6 min; Brinkmann Instruments, Inc., Westbury, N. Y.) the adsorption of radioactive IgG to the membranes was determined and calculated as percentage of total radioactivity. No unspecific binding of radiolabeled antibodies to control, untreated cells, or untreated membranes was observed.

Commercially available anti-staphylococcal α-toxin was purchased from Wellcome Laboratories (Beckenham, England) and from the Behringwerke. Antibodies to human serum protein components and to human erythrocyte membrane proteins (code A 104) were obtained from Dakopatts, Copenhagen, Denmark.

Quantitative Immunoelectrophoresis

Crossed immunoelectrophoresis, fused rocket immunoelectrophoresis and double-diffusion analyses were performed in 1% agarose gels (Litex, Glostrup, Denmark; type HSA) as described (10). In all the depicted experiments, the agarose used in first-dimension electrophoresis as well as in the application strip in fused rocket immunoelectrophoresis contained 0.5% (vol/vol) Triton X-100 + 0.2% (wt/vol) DOC. The presence of DOC increased the electrophoretic mobility of α-toxin and improved the resolutions. The antibody-containing agarose contained 0.5% Triton X-100 and no DOC in all cases. Detergent was not added to the electrophoresis buffer (0.1 M glycine, 0.038 M Tris, pH 8.7). Charge-shift crossed immunoelectrophoresis was performed as described (9).

SDS PAGE

SDS PAGE was performed in gel rods (5 mm diameter) according to Fairbanks et al. (23). Discontinuous slab gels (1.5 mm thickness) based on the Laemmli procedure (37) and prepared as described (13) were used to monitor protein separations. Molecular weight determinations were performed in the continuous gel system using 4.4 and 7.5% gels. Calibrations were done with standard proteins from Pharmacia (phosphorylase b, 94,000 mol wt; bovine serum albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 20,100; α-lactalbumin, 14,400).

Assays for Hemolytic Activity and Neutralization Tests

Hemolytic activity of samples of native α-toxin was assayed by incubation

with 1 vol of a 2.5% rabbit erythrocyte suspension in phosphate-buffered saline, pH 7.3, for 30 min at 37°C. Hemolysis was read visually. Hemolytic titers were estimated by serial dilution of the samples with saline in the conventional manner.

Antisera raised against α-toxin were tested for toxin-neutralizing activity. Dilutions of the antiserum in phosphate-buffered saline were incubated with 1 U of α-toxin reagent (Behringwerke) for 30 min at 37°C. After addition of rabbit erythrocyte suspensions, the mixtures were incubated for another 30 min at 37°C. The end point was taken as the last tube in the series showing no hemolysis.

Electron Microscopy

Negative stainings and electron microscopy were performed as previously described (53).

Protein and Lipid Analyses

Protein concentrations were determined by amino acid analyses in all cases. Phosphorus was quantified by the method of Ferber et al. (24).

Ultracentrifugal Analysis of α-Toxin

The ultracentrifugal analyses were performed in model E apparatus equipped with UV-Vis-monochromator, scanner, and multiplexer for use of multicell rotor, type An-F Ti, with double sector cells (Beckman Instruments). Sedimentation velocity experiments were run for estimation of homogeneity of the preparation following the band-forming method of Kegeles (35) and Vinograd et al. (54). For high-speed performance (up to 60,000 rpm), aluminium double sector cells were modified and experimental conditions standardized as described by Kranz and Schmidt (36). Molecular weight runs were performed at 14,000 rpm and at 20°C following Yphantis (59). Both ultracentrifugal experiments were registrated and evaluated by on line computer coupling (type HP 1000; Hewlett-Packard, Palo Alto, Calif.), as will be published shortly.

Marker Release Studies

The capacity of α-toxin to generate circumscribed lesions in target membranes was examined by trapping radioactive markers of defined dimensions into resealed erythrocyte ghosts (28, 49, 50) and studying their release upon addition of the toxin in lytic concentrations. The following markers were utilized: myoglobin from horse (17,800 mol wt; Serva; effective diameter, 40 Å [45]); [³H]inulin (5,200 mol wt; Amersham Buchler (Braunschweig GER); diameter 30 Å [42, 43]); [³H]sucrose (243 mol wt; Amersham Buchler; diameter 8.8 Å [42,43]). Myoglobin was labeled with ^{125}I by the chloramine-T technique (29).

For reasons unclear at present, we were unable to produce stable, resealed rabbit erythrocyte ghosts, and human and sheep erythrocyte membranes were therefore utilized in these experiments. A 50% suspension of washed erythrocytes in 0.15 M Veronal-buffered saline (VBS), pH 7.3, was hypotonically hemolysed (final osmolarity, 30 mM VBS, 4 mM MgCl₂) in the presence of radioactive marker molecules at 0°C. After 20 s, reisotonization was achieved by addition of 0.75 M VBS, pH 7.3, and membranes were stabilized by subsequent incubation for 1 h at 37°C. After being washed three times in VBS, a 30% cell membrane suspension was layered over 43% sucrose in 25 mM NaCl, 25 mM Tris, pH 7.4 (14). Resealed ghosts were separated from nonresealed ghosts by centrifugation for 60 min at 30,000 rpm, rotor SW 41. The resealed ghosts floating on top of the sucrose solution were harvested and washed in VBS. The ghosts retained 80–90% of the entrapped radioactive markers over a tested period of 72 h at 4°C.

Ghost suspensions were brought to 20% and treated with increasing amounts of purified α-toxin for 30 min at 37°C. After incubation, ghosts were sedimented by centrifugation (Eppendorf centrifuge, 12,000 g × 6 min) and the degree of marker release was assayed by measuring the radioactivity present in the supernate and the pellets. Radioactivity was determined and calculated as percentage of radioactivity released in the supernate of a saponin-treated control. Quench for ³H-labeled samples was corrected by use of an internal standard.

In parallel with the marker release experiments, another set of ghost samples not carrying radioactive markers was treated with α-toxin, and binding of α-toxin was followed by determining the binding of radioactively labeled immunoglobulins to the membranes. A third assay was performed with a 20% suspension of intact erythrocytes to obtain an approximate correlation between the degree of hemolysis as a function of α-toxin concentration.

RESULTS

In ultracentrifugal analyses, the major protein component representing native α-toxin accounted for nearly 98% (wt/wt) of total sedimenting material. The sedimentation coefficient $S_{20,w}$

was found to be 3.3S, and a molecular weight of 33,900 (±4,700) was determined at sedimentation equilibrium. In SDS polyacrylamide gels, a protein band of apparent molecular weight 34,000 was found migrating between ovalbumin (43,000 mol wt) and carbonic anhydrase (30,000 mol wt) (see Fig. 1) that accounted for 95% of Coomassie-Blue–stainable material. The total amino acid composition of the protein (not shown) was in close agreement with that given by Six and Harshman (47, 48). Toxin concentrations of 0.35 μg/ml and 35–50 μg/ml caused 100% hemolysis of 2.5% suspensions of rabbit and human erythrocytes, respectively. Hemolysis could be inhibited by the use of commercial antisera to α-toxin (Wellcome Laboratories and the Behringwerke), as well as by antisera raised against the purified, membrane-derived toxin (see below).

Isolation of Membrane-bound α-Toxin

Both α-toxin in its purified form and α-toxin contained in an unfractionated culture supernate were used as the source for isolating α-toxin from either rabbit or human target erythrocyte membranes. Purification of the same protein resulted in all cases, although recoveries were low when culture supernates were applied, because of extensive membrane vesiculation. The use of unfractionated culture supernates also led to extensive degradation of erythrocyte membrane proteins (but not to the
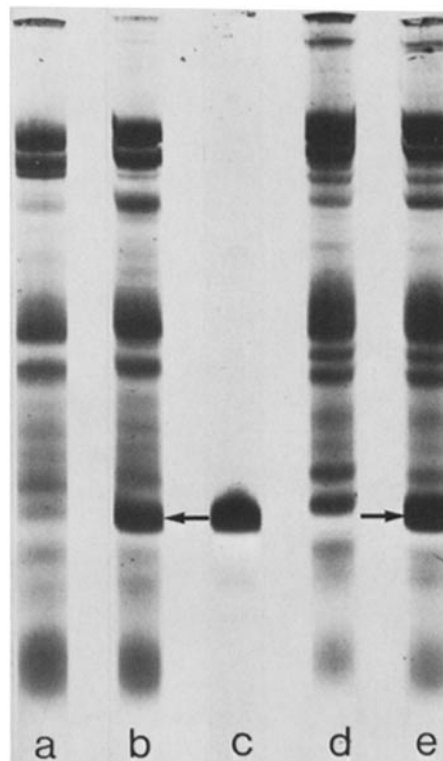

FIGURE 1 SDS electrophoresis in 4.4% polyacrylamide gels of control, hypotonically lysed rabbit (a) and human (d) erythrocyte membranes, α-toxin-treated rabbit (b) and human (e) erythrocyte membranes, and native α-toxin (c). Samples applied to gels a, b, d, and e were boiled in 2% SDS before electrophoresis. The sample of native toxin (gel c) was incubated in SDS at room temperature before electrophoresis. Toxin-lysed membranes exhibited unaltered polypeptide patterns compared to the controls, apart from the appearance of an additional polypeptide band that migrated identically as native α-toxin (arrow). Direction of electrophoresis: top to bottom.

protease-resistant, membrane-bound α-toxin, see below). The following section describes the isolation of membrane-bound toxin from erythrocytes lysed with purified α-toxin.

Fig. 1 depicts SDS polyacrylamide gels of control, hypotonically lysed rabbit and human erythrocyte membranes (gels *a* and *d*). The pattern of human erythrocyte membrane proteins was as described by Fairbanks et al. (23). Rabbit or human erythrocyte membranes that had been treated with α-toxin (gels *b* and *e*) exhibited the same polypeptide pattern, apart from the additional appearance of a protein band of 34,000 mol wt (arrow) that corresponded positionally to the band found upon electrophoresis of purified, native α-toxin (gel *c*). All membrane samples were boiled in SDS before electrophoresis. Reduction with 40 mM dithiothreitol (DTT) did not affect any of the protein patterns.

The amount of α-toxin that became bound to the membranes during incubation was estimated by hemolytic titration of α-toxin remaining in the supernate, as well as by densitometry of the 34,000 mol wt band in SDS gels obtained by electrophoresis of the α-toxin preparation before and after incubation with washed erythrocyte membranes. The results indicated that ~80% of the toxin became membrane-bound and no significant differences in the binding capacity for α-toxin between rabbit and human erythrocytes were found under the present experimental conditions.

The elutability of the 34,000 mol wt protein, assumed to represent membrane-bound α-toxin, was tested by treating the washed membranes with various buffers as detailed in Materials and Methods. The extent of elution of the protein was estimated by densitometry of the electrophoretograms obtained after electrophoresis of the aqueous supernates and of the membrane pellets. None of the tested eluents liberated significant amounts of the 34,000 mol wt protein (data not shown).

The failure to liberate substantial amounts of membrane-bound α-toxin by ionic manipulations was confirmed by immunoelectrophoresis experiments with the use of an anti-toxin antiserum. In these experiments, equivalent amounts of the buffer eluates and the corresponding, extracted membrane pellets were applied in wells in agarose gels and subjected to rocket immunoelectrophoresis in the presence of detergent. Over 90% of the protein immunologically identifiable as α-toxin remained associated with the membrane pellets in every case. Identical results were obtained with both rabbit and human erythrocytes (results not depicted).

Fig. 2 (lower panel) shows SDS gel patterns of native α-toxin (gel *a*), toxin-treated rabbit erythrocyte membranes (gel *b*), a 1% Triton extract of the membranes (gel *c*), and the Triton-extracted pellet of the membranes (gel *d*), obtained in a discon-
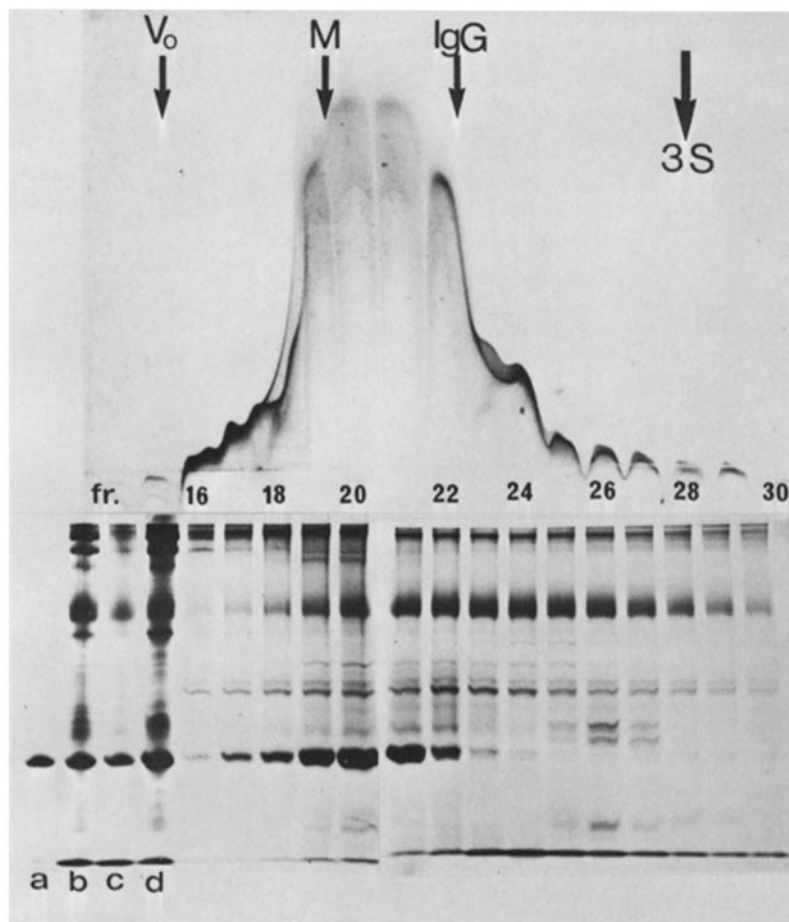

FIGURE 2 Elution profile of Triton-extracted, α-toxin-treated rabbit erythrocyte membrane proteins upon Sephacryl S-300 chromatography, monitored by rocket immunoelectrophoresis and SDS gel electrophoresis. SDS gels *a–d* were loaded with: native α-toxin (*a*), toxin-treated membranes (*b*), Triton extract of toxin-treated membranes (*c*), and the unsolubilized Triton membrane residue (*d*). A detergent extract (polypeptide pattern of gel *c*) was chromatographed over Sephacryl S-300 in the presence of 2.4 mM Triton X-100. Aliquots of each column fraction were analyzed by rocket immunoelectrophoresis (top part of figure) with the use of an antiserum to α-toxin, and by SDS gel electrophoresis (bottom part of figure). Eluting positions of α_2-macroglobulin (*M*), human serum IgG, and native 3S toxin on the same column are indicated; V_0: column void eluting volume.

tinuous gel electrophoresis system. Triton preferentially solubilized erythrocyte membrane protein band 3 and the protein band positionally corresponding to α-toxin. The degree of solubilization of the 34,000 mol wt band by 1% Triton was estimated to be ~35–40% by densitometry. When Triton extracts of membranes containing the proteins depicted in Fig. 2, gel b, were chromatographed over a Sephacryl S-300 column equilibrated with 2.4 mM Triton X-100, the protein band corresponding to α-toxin was found eluting symmetrically at a position intermediate between those of α_2-macroglobulin and IgG. Parallel immunoelectrophoresis using antiserum to the purified, membrane-derived toxin permitted immunological detection of this component in the same fractions (Fig. 2, upper panel). The eluting position of native, 3S toxin on the same column is indicated.

Fractions from the column containing the 34,000 mol wt protein as identified by SDS PAGE were pooled, concentrated, and applied to linear 10–43% sucrose density gradients containing 1.2 mM Triton X-100. After ultracentrifugation, the bulk of the 34,000 mol wt protein, corresponding immunologically to α-toxin (Fig. 3), was recovered in a symmetrically sedimenting 11S peak (see Fig. 7 below) and pooled for membrane reconstitution experiments.

The final purification step consisted of reincorporation of the 34,000 mol wt protein into bilayers of α-lecithin. Fractions containing the protein were added to a solution of α-lecithin/DOC at protein/lipid ratios of 1:20–1:30 (wt/wt). After removal of detergent through dialysis, liposomes formed that were floated through sucrose solution. In control experiments, wherein the 34,000 mol wt protein was dialyzed without addi-

tion of lipid, all the protein remained at the bottom of the centrifugation tubes during centrifugation (Fig. 4A). If, however, lipid was present during the dialysis, a part of the protein became lipid-associated and floated through the sucrose solution (Fig. 4B). The final distribution of protein in the sucrose gradients varied in different experiments, because of the heterogeneity of the protein-lipid aggregates that had formed during dialysis. The top fractions recovered from such gradients contained protein/lipid ratios ranging from 1:30 to 1:60 (wt/wt) and were utilized for electron microscopy, immunization, and SDS PAGE. A single protein band, corresponding to that of native α-toxin, was found upon SDS PAGE (Fig. 4C, gels a and b). If the sample was not boiled in SDS before electrophoresis, a single major band of apparent molecular weight 200,000 was found instead of the 34,000 mol wt band. When the 200,000 mol wt band (Fig. 4, gel c) was sliced from gels, heated to 90°C for 1 min, and reelectrophoresed, the 34,000 mol wt band resulted (results not depicted). Thus, the 200,000 mol wt band apparently represents an oligomer of the 34,000 mol wt protein that requires heating in the presence of SDS for dissociation and monomerization. In contrast, native α-toxin did not require heating in SDS for generation of the 34,000 mol wt band. The same electrophoretic behavior has been reported for α-toxin oligomers in other laboratories previously (20).

Attempts to elute the 34,000 mol wt protein from reconstituted liposomes with various salt solutions were uniformly unsuccessful, <10% release being detectable by any one of the tested eluents (results not shown).

Characterization and Identification of the Isolated Protein as α-Toxin

The isolated protein was identified as α-toxin on the basis of

FIGURE 3 Centrifugation of pooled and concentrated, α-toxin-containing fractions from the Sephacryl column in 10–43% (wt/wt) sucrose density gradients containing 1.2 mM Triton X-100. Aliquots of fractions from the gradient were analysed by SDS PAGE (lower part of figure) and by rocket immunoelectrophoresis using anti-α-toxin antiserum (upper part of figure). A reference sample of native 3S α-toxin was electrophoresed in gel a.
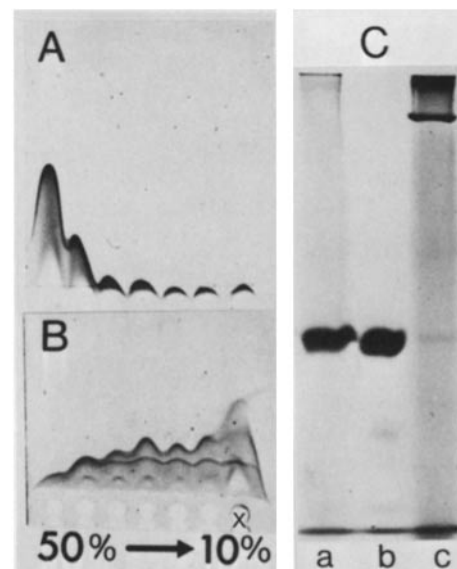

FIGURE 4 Preparations of membrane-derived α-toxin, dialyzed in the absence (A) or presence (B) of α-lecithin/DOC, were applied to the bottom of a sucrose gradient. After centrifugation, all the protein in the control preparation A remained at the bottom of the gradient, whereas a major part of the protein in experiment B floated through sucrose in lipid-associated form. The direction of the sucrose gradient is given. α-Toxin was detected in the fractions by rocket immunoelectrophoresis. (C) SDS PAGE of the top fraction recovered from the sucrose flotation gradient of B: (a) liposome sample boiled in SDS before electrophoresis, (b) reference native 3S α-toxin, (c) SDS-treated, but unheated liposome sample.

its immunological, biochemical and ultrastructural properties.

IMMUNOLOGICAL PROPERTIES: When rabbits were immunized with the protein isolated from either rabbit or human erythrocytes, antisera were obtained that precipitated only one protein in a crossed immunoelectrophoresis of a crude detergent extract of toxin-treated rabbit or human erythrocyte membranes (Fig. 5 B). These antisera did not precipitate any membrane protein from native erythrocytes. They exhibited toxin-neutralizing titers of 128-256 when tested with commercially available α-toxin reagents. In double diffusion, the isolated protein was precipitated both by the specific antiserum raised in this laboratory and by a commercial anti-α-toxin serum (Fig. 5 A). The protein was not precipitated by antibodies to human erythrocyte membranes.

When an immunoprecipitate obtained by a crossed immunoelectrophoresis as depicted in Fig. 5 B was extensively washed, sectioned from the agarose plates, boiled in 2% SDS, and electrophoresed in polyacrylamide, the 34,000 mol wt protein band was found in addition to a 160,000 mol wt immunoglobulin band (results not shown), confirming the identity of the precipitated protein with the 34,000 mol wt protein.

BIOCHEMICAL PROPERTIES: The isolated protein exhibited the same amino acid composition as native α-toxin. As shown above, it was also electrophoretically identical to native α-toxin. When liposome preparations were treated with trypsin, chymotrypsin, or pronase at enzyme/protein ratios of 1:50 (wt/wt) for 3-16 h at 22-37°C, no degradation of the 34,000 mol wt protein whatsoever could be discerned. Treatment with 40

mM DTT, alone or combined with protease treatment, also did not alter the electrophoretic banding behavior of the protein. The extreme resistance of oligomerized α-toxin toward proteolytic degradation has been reported previously (20).

The resistance of α-toxin against elution from target membranes through ionic manipulations, and the capacity of the protein to bind lipid both indicated an amphiphilic nature of the membrane-bound toxin. This contention was subsequently corroborated by the demonstration of detergent binding through charge-shift electrophoresis, and by the finding that the isolated protein aggregated in aqueous solution after removal of detergent.

Fig. 6 depicts a charge-shift crossed immunoelectrophoresis of the isolated protein. Binding of detergent was indicated from the pronounced bidirectional "charge shifts" induced by the presence of DOC (plate A) and by cetyltrimethylammonium-bromide (CTAB) (plate C), as compared with the electrophoretic mobility of the protein in the presence of Triton X-100 alone (plate B; see references 9 and 33 for details). The behavior of membrane-derived α-toxin in charge-shift electrophoresis was thus characteristic of an amphiphilic detergent-binding protein.

When liposomes reconstituted with α-toxin were solubilized in 1% Triton and applied to linear sucrose density gradients containing 1.2 mM Triton X-100, the detergent-protein complex resedimented in sucrose with a sedimentation coefficient of 11S ± 0.5 (Fig. 7A), identically as also shown in Fig. 3. Fractions containing the isolated protein were pooled and dialyzed for 96 h at room temperature to remove detergent. Phosphorus analyses failed to reveal the presence of lipid in these fractions, indicating that the protein had been recovered in extensively delipidated form. When a dialyzed sample was resedimented in a sucrose density gradient lacking detergent, the protein sedimented as an aggregate and was recovered in the bottom fractions of the sucrose gradient (Fig. 7B). If, on the other hand, the protein was dialyzed in the presence of 2.4 mM detergent over the same period, it retained its sedimentation coefficient of 11S and sedimented identically as depicted in Fig. 7A. The membrane-derived form of α-toxin thus further
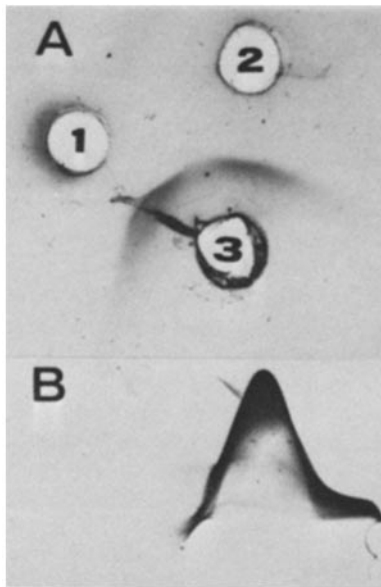

FIGURE 5 (A) Double-diffusion analysis of liposomes carrying α-toxin (well 3), using an antiserum raised against the toxin-laden vesicles (1) and anti-α-toxin from Wellcome Laboratories (2). The agarose gel contained 0.5% Triton X-100. (B) Rabbit erythrocyte membranes treated with α-toxin contained in an unfractionated bacterial culture supernate were solubilized in Triton and analyzed by crossed immunoelectrophoresis using antiserum raised against α-toxin-loaded liposomes (4 μl/cm²). First-dimension electrophoresis: 10 V/cm × 45 min. The first-dimension agarose gel contained 0.5% Triton + 0.2% DOC. Second-dimension immunoelectrophoresis: 2 V/cm for 16 h. The agarose gel contained 0.5% Triton. The antiserum raised against the purified protein precipitated only one protein in the detergent extract, corresponding to membrane-bound α-toxin.
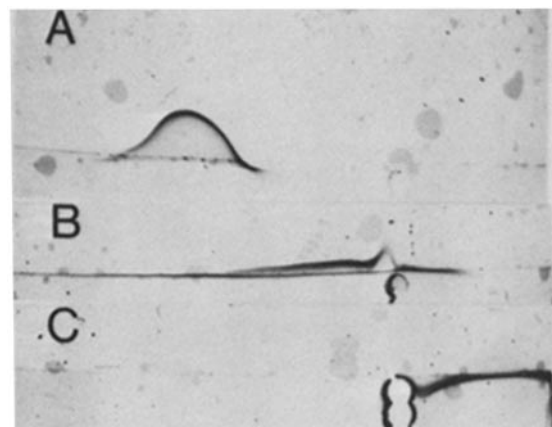

FIGURE 6 Charge-shift crossed immunoelectrophoresis of a Triton-extract of toxin-treated rabbit erythrocyte membranes. First-dimension electrophoresis was performed in gels containing Triton (plate B), Triton + DOC (plate A) and Triton + CTAB (plate C) for 4 h at 5 V/cm. Second-dimension immunoelectrophoresis was performed at 2 V/cm × 16 h in gels containing 0.5% Triton and 4 μl/cm² anti-α-toxin antiserum. Membrane-bound α-toxin exhibited pronounced bidirectional charge shifts (see references 10 and 33) characteristic of an amphiphilic, detergent-binding protein.
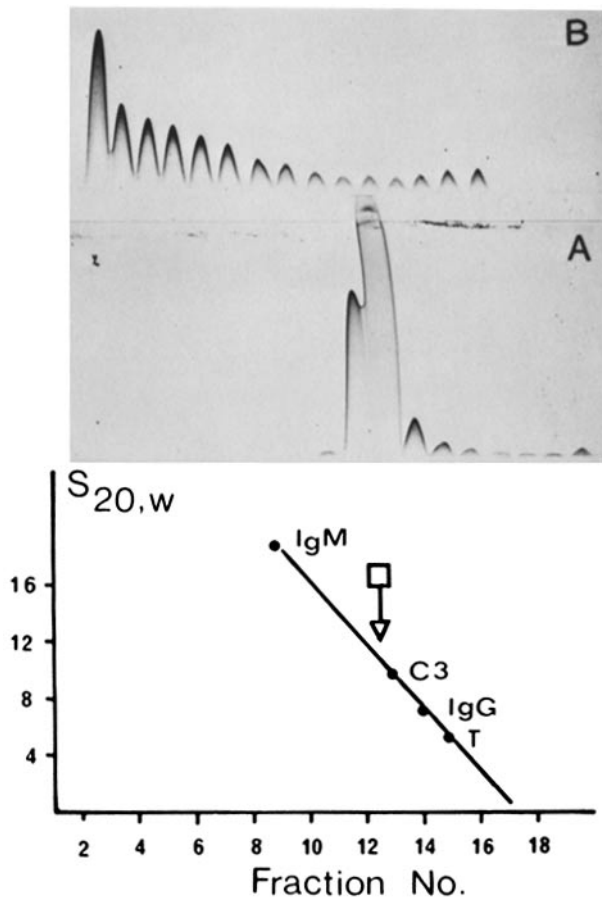
Figure 7 Sedimentation behavior of α-toxin extracted from liposomes in a sucrose density gradient containing 1.2 mM Triton X-100 (A). After dialysis for 96 h to remove detergent, the sample was resedimented in a density gradient lacking detergent (B). The positions of the protein in the gradients were determined by fused rocket immunoelectrophoresis using an antiserum to α-toxin. Sedimentation positions of IgM (19S), C3 (9.5S), IgG (7S), and transferrin (5.5S) were plotted against sedimentation coefficients ($S_{20,w}$) to approximate the sedimentation coefficient of α-toxin in Triton solution. Membrane-derived α-toxin exhibited an apparent sedimentation coefficient of 11S in Triton (A). Removal of detergent led to aggregation of the protein (B).

resembled classical "integral" membrane proteins in its tendency to aggregate in aqueous solution in the absence of detergents.

Dialysed toxin preparations were also tested for hemolytic activity toward erythrocytes. At protein concentrations of 0.2 mg/ml, no hemolytic activity could be detected with the use of rabbit erythrocytes.

ULTRASTRUCTURE OF MEMBRANE-DERIVED α-TOXIN: Negative stainings of native 3S toxin revealed the presence of finely granular material only (Fig. 8 C). Intact rabbit or human erythrocytes incubated with *S. aureus* culture supernate yielded membranes that were fragmented into 0.1- to 0.6-μm vesicles, carrying distinct ring-shaped structures (Fig. 8 A). Erythrocytes lysed with purified toxin did not vesiculate appreciably and carried the same structures on their surface (Fig. 8 B). Along the sharply bent edges of the negatively stained ghosts, small stubs were seen in profile, projecting ~5 nm outside the plane of the membrane. These structures entirely resisted proteolytic degradation.

Purified toxin isolated from target membranes and delipi-

dated through centrifugation in sucrose density gradients containing Triton were viewed in negative stainings as small rings. Stained with sodium silicotungstate, their outer diameter measured ~8.5 nm (Fig. 8 D). In stainings with uranyl acetate, the rings appeared significantly larger, with an outer diameter of 10 nm (Fig. 8 E). The same difference in outer diameter of the rings was observed on similarly stained, toxin-treated membranes. The internal diameter of the rings, most clearly visible in stainings with uranyl acetate, was ~2.5 nm (Fig. 8 F).

Lipid vesicles formed in the presence of α-toxin and subsequently isolated by flotation exhibited structures indistinguishable from the rings and stubs observed on membranes lysed with the toxin. In 10 individual experiments, the liposomes were reconstituted asymmetrically, with the toxin molecules projecting external to the lipid vesicles (Fig. 8 G). Typically, liposomes carrying rings and stubs were readily permeable to the negative stain, whereas liposomes devoid of such structures did not take up the silicotungstate stain. These findings may indicate increased permeability of the liposomal bilayer to the negative stain after incorporation of α-toxin (Fig. 8 H).

Marker Release Studies

The collective data presented above raised the possibility that upon binding to a target membrane, α-toxin molecules might self-associate to form ring-structured oligomers that, through exposure of apolar surfaces, would become partially embedded within the lipid bilayer to generate an aqueous, transmembrane pore. Should this model of toxin-membrane interaction be correct, the toxin-dependent lesion would be expected to be discrete in size.

This possibility was examined through the study of toxin-dependent release of defined molecular markers after their entrapment within resealed erythrocyte ghosts. In parallel, radioactive antiglobulin binding assays were used to follow membrane binding of α-toxin, and lysis of intact erythrocytes was also followed to obtain an approximate correlation between marker release and hemolysis.

Fig. 9 A depicts a marker release study conducted with resealed human erythrocyte ghosts. In the presence of buffer, without toxin, ~90% of the markers remained entrapped in the cells. When α-toxin was added in increasing concentrations, liberation of the small marker sucrose paralleled the extent of hemolysis and was complete at a toxin concentration of 35 μg/ml with human and 7 μg/ml with sheep erythrocyte ghosts (Fig. 9 B). At toxin concentrations causing partial hemolysis, the partial sucrose release depicted in Fig. 9 A and B represented plateau values that did not increase upon prolonged incubation periods (up to 3 h). Release of inulin (effective molecular diameter, 30 Å) occurred with some retardation. The toxin concentration causing 100% hemolysis or 100% sucrose release liberated 60% inulin from human and 20% from sheep erythrocyte ghosts after a 30-min incubation. In both cases, inulin release was complete at 70 μg/ml toxin concentration. Myoglobin (effective diameter, 40 Å) was not released from resealed human or sheep erythrocyte ghosts at maximal toxin concentrations tested (70 μg/ml).

These results indicated that α-toxin induces membrane lesions that allowed free passage of small molecules, whereas diffusion of larger molecules (myoglobin) was totally restricted. In an earlier study it was similarly found that human serum albumin (effective diameter, 72 Å) was also not released from resealed ghosts through the action of α-toxin (49). Release of inulin was retarded possibly because of its marginal size. In a
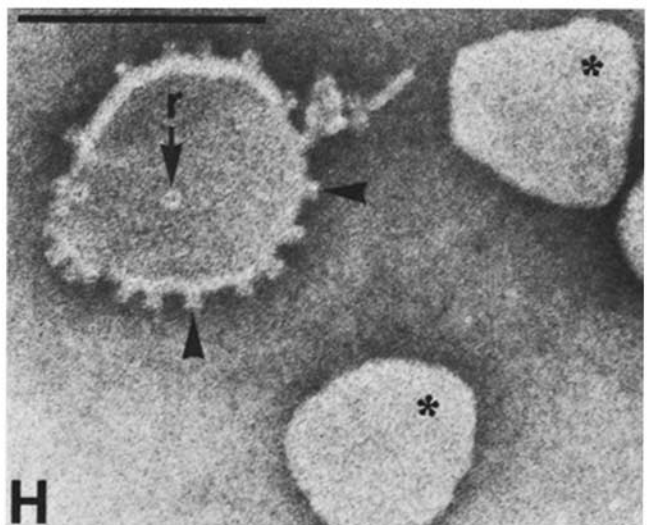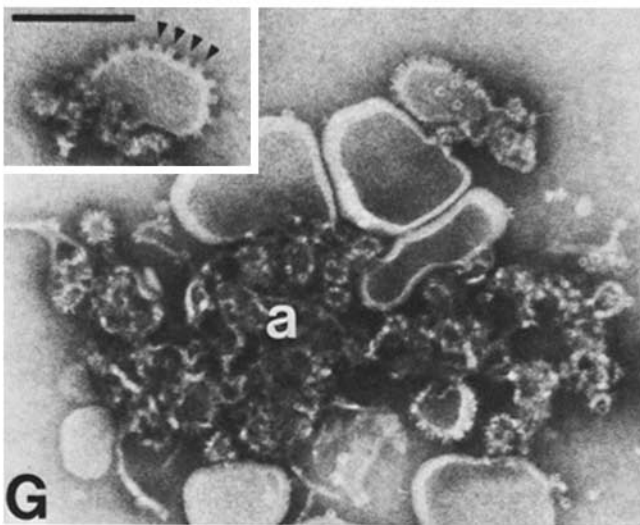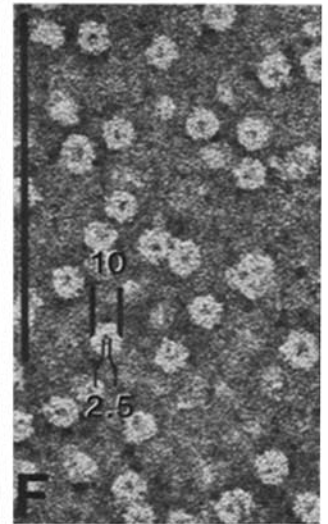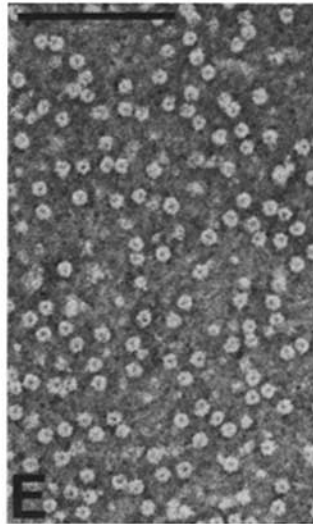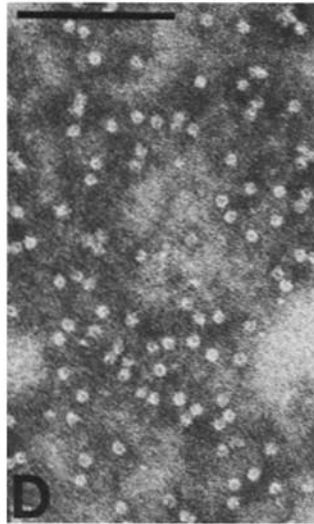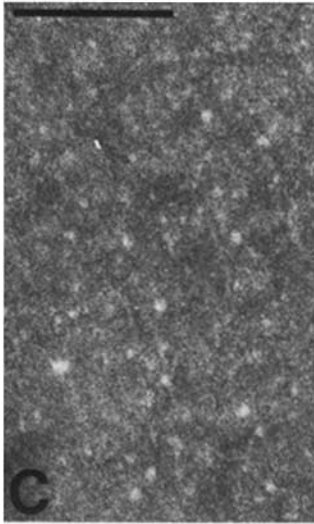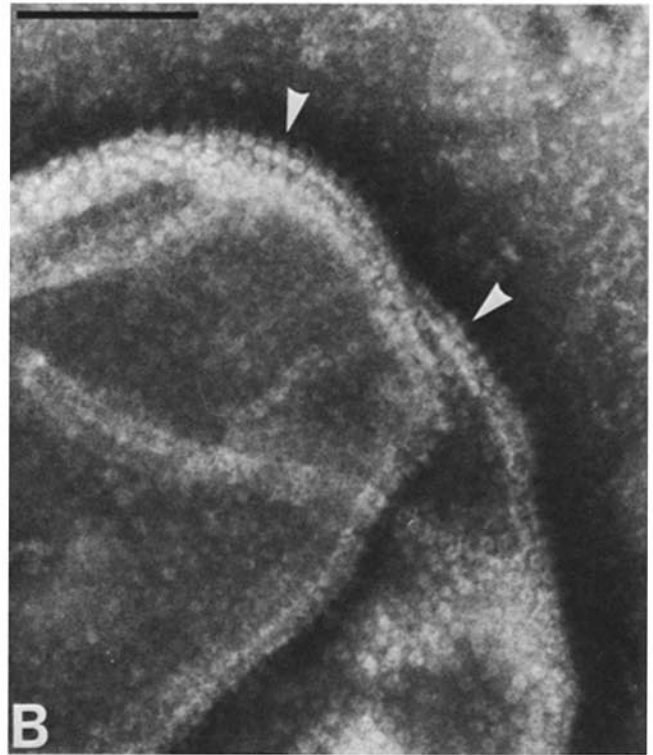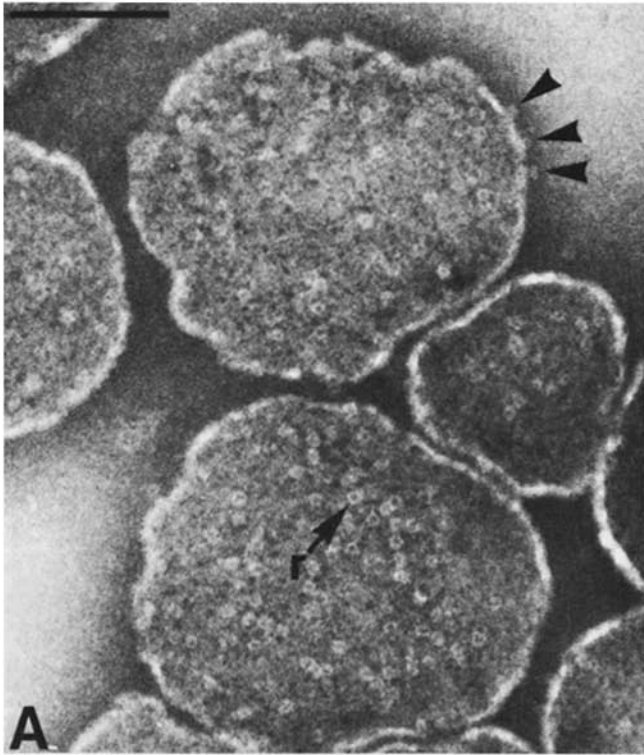
further experiment, release of inulin was observed over a period of 24 h. Human erythrocyte ghosts were incubated for 30 min with 30 μg/ml α-toxin. Cells were washed three times by centrifugation, resuspended in isotonic buffer, and kept at 37°C. In intervals, samples were withdrawn and release of inulin was measured. Ghosts containing entrapped myoglobin, and incubated with buffer clone, served as controls.

After the first incubation period of 30 min, α-toxin treatment caused 29% release of inulin. During the following 24 h a continuous release of inulin was observed, whereas myoglobin remained entrapped in ghosts to the same extent as both markers in the buffer controls (Fig. 9 C). Identical results were obtained in experiments with resealed sheep erythrocyte ghosts (not shown).

A radioactive antiglobulin binding assay was additionally utilized to follow the binding of the toxin to the cell membranes. In contrast to the difference in hemolysis and marker release, the amount of membrane-bound α-toxin appeared to be approximately equivalent in these assays.

DISCUSSION

After treatment of erythrocyte membranes with S. aureus α-toxin, a protein has been isolated from membrane detergent solubilizates that, by virtue of its biochemical, immunological, and ultrastructural properties, has been shown to represent the membrane-bound form of α-toxin.

By analytical ultracentrifugation and SDS PAGE, native α-toxin was found to be a water-soluble molecule with a molecular weight of 34,000. This value agreed with earlier determinations of McNiven et al. (41) and Watanabe and Kato (55), but disagreed with the value of 28,000 obtained by Six and Harshman (47, 48). In contrast, α-toxin isolated from target membranes was a large molecule eluting in detergent gel chromatography at a position intermediate between those of IgG and α_2-macroglobulin, and possessing a sedimentation coefficient of 11S. In the electron microscope, native α-toxin was visualized as a finely granular material, whereas the membrane-derived toxin was observed as a ring structure harboring a central pore, identical to the ring structures visible on toxin-lysed erythrocyte membranes. Because the toxin molecules projected as short stubs from the edges of the membrane, an overall cylindrical structure was indicated. The measurable outer diameter of the molecule was larger (10 nm) in staining with uranyl acetate compared with staining with silicotungstate (8.5 nm), an effect possibly attributable to the low pH (3.7) to which the toxin was exposed during uranyl acetate staining. Similar deviations in the dimensions of the rings have been

observed earlier (3, 25).

A hollow protein cylinder with a partial specific volume of 0.72 (determined from the amino acid composition of α-toxin), 5 nm in height, with outer and inner diameters of 8.5 and 2.5 nm, respectively, would have a molecular weight of ~200,000, equivalent to a toxin hexamer. This value would be consistent with the appearance of the 200,000 mol wt protein band in SDS gels. Recent hydrodynamic data have indeed confirmed the molecular weight of 200,000 for the toxin rings (S. Bhakdi, R. Füssle, and J. Tranum-Jensen, manuscript submitted for publication).

We thus conclude that, as originally proposed by Freer et al. (25, 26), membrane binding of toxin is accompanied by oligomerization of the native molecules to form ring-shaped complexes. Because we were unable to detect significant amounts of any other membrane protein or lipid component in purified toxin preparations, the rings appeared to be composed solely of oligomerized α-toxin. In contrast Cassidy and Harshman (20), we were not able to detect the presence of other, higher molecular weight forms of detergent-solubilized toxin, by either sucrose density gradient ultracentrifugation or gel chromatography. We were also not able to detect substantial amounts of low molecular weight toxin forms in membrane-detergent solubilizates that corresponded in their eluting positions to native 3.3S toxin in gel chromatography. Virtually all the membrane-bound toxin was found to be present in 11S form, and it appears probable that the process of complex formation therefore represents the primary feature of toxin-membrane interaction that is directly linked to its lytic property.

Membrane-derived toxin exhibited properties that were entirely comparable to those of classic integral membrane proteins (15, 32, 46, 51). The protein was poorly eluted from membranes through ionic manipulations. Our data may stand in some conflict with earlier results of Cassidy and Harshman (19), who reported elution of radioiodinated, membrane-bound toxin with chaotropic salts. In the present study the degree of extraction of α-toxin from membranes and liposomes was evaluated by SDS PAGE and by rocket immunoelectrophoresis. Whereas these methods are not as sensitive as radioassays, they possess the advantage that the protein is unmanipulated. By contrast, radioiodination affects the lytic capacity of toxin (18, 31), and it is therefore conceivable that the radioiodinated toxin (19) exhibited somewhat deviating properties in membrane-binding and elutability as compared with those of the unlabeled molecule.

Direct evidence for an amphiphilic nature of membrane-derived α-toxin was obtained by charge-shift electrophoresis and membrane reconstitution experiments. The former tech-

FIGURE 8 (A) Vesicular fragments of rabbit erythrocytes lysed with Staphylococcus culture supernate seen in negative staining with sodium silicotungstate. Ring-shaped structures (r) are seen over the membranes, and small stubs (arrowheads) project from the sharply bent edges of the ghost membranes. X 203,000. (B) Rabbit erythrocyte, prepared as in A, but with purified 3S α-toxin. The same ring-shaped and stubby structures appear in quantity, but the membranes do not vesiculate. X 230,000. (C) Purified 3S α-toxin, negatively stained with sodium silicotungstate. Only a finely granular structure appears. X 203,000. (D) Highly purified α-toxin in 11S form, negatively stained with sodium silicotungstate. The molecules appear as ring-shaped structures of 8.5 nm outer diameter, surrounding a central stain deposit. X 203,000. (E) The same 11S α-toxin preparation as in D, negatively stained with uranyl acetate. In this staining, the outer diameter of the molecule is consistantly larger, viz. 10 nm. A similar preparation is shown at high magnification (X 453,000) in F. The diameter of the central pit/channel measures 2.5 nm. (G and H) Liposomes formed in the presence of purified 11S α-toxin, which has become incorporated into the liposomal membrane to appear as rings (r) and stubs (arrowheads). Liposomes devoid of incorporated toxin (asterisks) are typically empty of stain, whereas liposomes carrying the toxin are permeable, whereby the liposomal membrane becomes visible as a light rim. G contains clusters of small liposomes carrying numerous α-toxin complexes (a) in addition to larger liposomes carrying fewer complexes. Sodium silicotungstate staining. G, X 163,000; H, X 325,000. Bars, 100 nm.
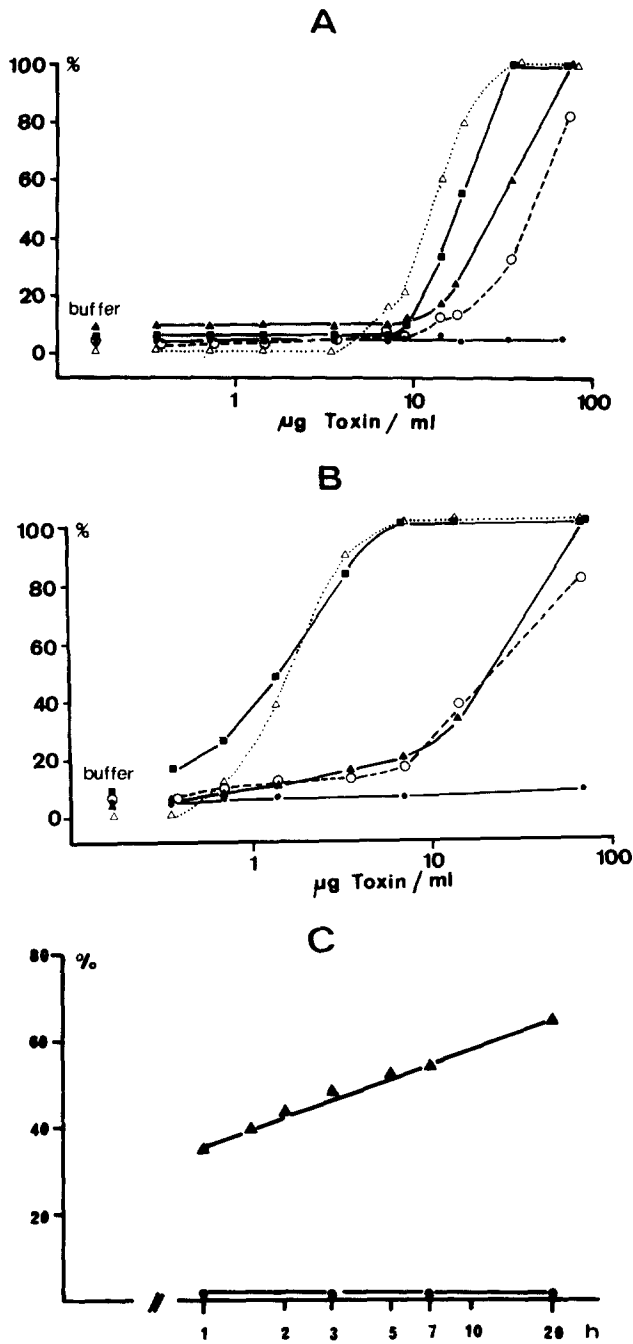
A

B

C

FIGURE 9 Selective release of entrapped marker molecules from resealed human (A) and sheep (B) erythrocyte ghosts by α-toxin. Resealed ghosts were treated with increasing amounts of α-toxin. The release of ¹²⁵I-myoglobin (●), ³H-inulin (▲) and ³H-sucrose (■) after a 30-min incubation, assayed as radioactivity in the supernates, was calculated as percent of radioactivity compared with the supernate of a saponin-treated control. Membrane-binding of α-toxin was followed by determining the binding of radioactively labeled, anti-α-toxin immunoglobulins (○) to the membranes. Hemoglobin release (△) was measured photometrically at 546 nm in a parallel experiment with intact erythrocytes. Lytic doses of α-toxin selectively released molecular markers whose effective diameter did not exceed 30 Å from the resealed ghosts. The depicted partial sucrose release effected by low toxin concentrations represented plateau values that remained constant over an incubation period of 3 h. (C) Human erythrocyte ghosts were incubated for 30 min with 30 μg/ml α-toxin, washed three times by centrifugation, and resuspended in VBS. During the subsequent storage, aliquots were withdrawn at intervals and the release of ³H-inulin (▲) and ¹²⁵I-myoglo-

nique yielded evidence for the presence of apolar, Triton-binding surfaces on the toxin oligomer (33), and this could be confirmed through reincorporation of the toxin rings into bilayers of lecithin. Membrane-derived α-toxin also aggregated in aqueous solution after removal of detergent. The aggregated toxin no longer exhibited hemolytic properties, probably because the apolar surfaces had become concealed within the aggregates and were no longer available for membrane-binding.

Should the ring-structured oligomer of α-toxin indeed be partially embedded within the lipid bilayer, the central pit or channel might be involved in perturbing the membrane permeability barrier, possibly by generating circumscribed pores in the bilayer. Previous studies indicated that toxin-damaged membranes were leaky for small molecules (21, 52). The observation that α-toxin-carrying liposomes typically are leaky towards silicotungstate (~3,500 mol wt), whereas toxin-free liposomes are impermeable, also indicates a toxin-induced permeability defect. In an attempt to corroborate and extend these observations, we undertook marker release experiments conducted with resealed erythrocyte ghosts. The results indeed indicated the presence of toxin-dependent, circumscribed lesions in the membranes leading to selective release of marker molecules whose effective diameter did not exceed 30 Å. This value agreed well with the dimensions of the proposed pore as observed in the electron microscope.

A possible model for the mechanism by which α-toxin damages membranes thus conceives of α-toxin as being a primarily hydrophilic molecule that, through contact with an appropriate substrate such as the target lipid bilayer, spontaneously self-associates on and in the membrane to form an amphiphilic oligomeric complex. The membrane-associated complex has the structure of a thick-walled cylinder, harboring a central pore. The cylinder orients perpendicular to the membrane plane and penetrates at least partially into the lipid bilayer. The presently available data do not of course permit any estimates to be made regarding the depth of membrane penetration. However, even partial insertion of an aqueous channel through the external lipid monolayer would be expected to perturb the integrity of the membrane permeability barrier. An aqueous channel would thus ensue, conceivably representing the primary, toxin-dependent membrane permeability defect.

This model has been proposed to explain the nature of the primary membrane lesion induced by α-toxin. We hereby assume that the mode of toxin-membrane interaction is uniform and that the toxin recovered and purified from Triton extracts is representative for all the toxin molecules present on a lysed membrane. The model would then be compatible with all the experimental data presented in this study, and would also be in accordance with the bulk of data that has accumulated on α-toxin in earlier reports. It is consistent with the data of Freer et al. (4, 25, 26) that the primary target of toxin attack is the lipid bilayer. It conforms with the notion that, upon binding to the membrane, oligomerization of the toxin molecules occurs, leading to the formation of 200,000 mol wt, ring-shaped hexamers (25). It agrees with the concept that the toxin oligomer damages membranes through a tight association with the lipid matrix (25, 26) and provides a structural model for the mode of membrane perturbation. It would be consistent

pended in VBS. During the subsequent storage, aliquots were withdrawn at intervals and the release of ³H-inulin (▲) and ¹²⁵I-myoglo-

bin (●) was followed over a period of 24 h. The noted values are corrected for spontaneous release determined in buffer controls.

with the findings of Cassidy and Harshman that membrane-binding and damage to the bilayer follow a defined, dissectable sequence of events (19). The first is presumably represented by toxin-binding to the membrane (19), a process that may be mediated by specific receptors (19, 34). Thereafter, the toxin molecules oligomerize to form the membrane-penetrating ring structures. Ion leakage then probably ensues through the generated pore, leading to osmotic swelling and, finally, to hemoglobin release through osmotic lysis of the cell.

In confirmation of the findings of Freer et al. (26) we have found that these processes occur in the absence of detectable changes in membrane protein composition, as evaluated by SDS PAGE. The concept of α-toxin being a protease that cleaves membrane proteins (57) is therefore not supported by our data. We have also been unable to detect any differences in molecular weight between native toxin and the subunit of membrane-derived toxin in SDS PAGE. The contention that native α-toxin is a protoxin that requires cleavage at the membrane surface to yield an active toxin (57, 58) thus also does not receive support, although the cleavage and removal of a very small polypeptide cannot yet be excluded.

At present, we have no explanation to account for the variations in susceptibility to lysis among erythrocytes of different animal species (5). It is conceivable that these are attributable to complex factors related to the difference in composition and organization of membrane constituents. Thus, membrane repair mechanisms may exist that are more effective in human than in rabbit erythrocytes. Because liposomes formed from human erythrocyte membrane lipids are as susceptible to toxin-mediated damage as those formed from lipids of rabbit erythrocytes (21), a role for membrane proteins in these putative repair processes appears indicated. Further work is obviously necessary to resolve this problem.

Pertinent analogies appear to exist between the mode of toxin-induced membrane perturbation and immune cytolysis by complement (12, 39). In both cases, hydrophilic proteins, i.e., native α-toxin or C5b-C9 complement components, undergo conformational changes upon contact with a target lipid bilayer, self-associating to form protease-resistant, amphiphilic protein complexes (Fig. 10). Current evidence indicates that the terminal complement C5b-C9 complex is a hollow, cylindrical protein structure that, through its embedment within the lipid bilayer, generates a transmembrane channel (12, 39). It is tempting to assume that staphylococcal α-toxin damages the membrane through a basically similar mechanism. Both phenomena might thus reflect a common principle of membrane attack and perturbation, i.e., the hydrophilic-amphiphilic transition of alien protein molecules in a lipid bilayer with the formation of aqueous, transmembrane channels walled by the inserted polypeptide chains.
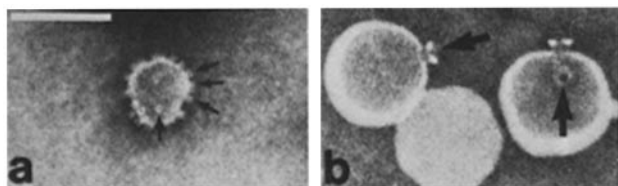

FIGURE 10 Electron micrographs of liposomes carrying reincorporated 11S α-toxin (a) and C5b-C9(m) complement complexes (b) (12). Arrows point to profile and en face views of both molecules, which appear to penetrate into the lipid bilayer to produce transmembrane channels. Bars, 100 nm.

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