

Quantitation of Monomeric and Oligomeric Forms of Membrane-Bound Staphylococcal Alpha-Toxin by Enzyme-Linked Immunosorbent Assay with a Neutralizing Monoclonal Antibody

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A murine monoclonal antibody generated against staphylococcal alpha-toxin was shown to react only with the monomeric (native), 3S form of the toxin. A sensitive sandwich enzyme-linked immunosorbent assay (ELISA) constructed with this antibody permitted detection of 0.25 to 0.5 ng of native toxin per ml. Toxin oligomers formed either by heat aggregation in solution, on target erythrocyte membranes, or on phosphatidylcholine-cholesterol liposomes were unreactive in the ELISA when membranes were solubilized with the nondenaturing detergent Triton X-100. After dissociation of the oligomers by boiling in sodium dodecyl sulfate, however, the ELISA reactivity of the liberated 3S toxin was fully restored. Parallel determinations of membrane-bound toxin with sodium dodecyl sulfate and Triton X-100 solubilization thus permitted direct quantitation of total and monomeric toxin, respectively; the difference between these two values was represented by toxin oligomers. The detection limits for membrane-bound oligomeric and monomeric toxin on erythrocyte membranes are in the order of 100 molecules and 1 molecule per cell, respectively. Using this ELISA, we show that over 90% of alpha-toxin molecules bound to target membranes at 37°C are in oligomeric form. Evidence is given that the monoclonal antibody neutralizes alpha-toxin by inhibiting its binding to both rabbit and human erythrocytes. This ELISA is the first assay that quantitatively discriminates between mono- and oligomeric forms of a pore-forming protein on target cell membranes.

Staphylococcal alpha-toxin is produced by most strains of *Staphylococcus aureus* and is considered an important determinant of bacterial pathogenicity (16, 19, 21). Early studies by Arbutnott et al. (1, 2) and Freer et al. (11, 12) identified the plasma membrane as the primary target of toxin action (1-4). These investigators concluded that the toxin, which is produced as a water-soluble, 3S polypeptide of M_r 34,000 (5, 19), undergoes a conformational change upon binding to a membrane that results in the formation of toxin hexamers. Since the toxin was able to damage liposomal bilayers (11) and lipid monolayers devoid of protein (9), it was initially thought that no specific membrane "receptor" was required for toxin action (19).

Subsequent work from this laboratory led to the isolation of alpha-toxin from target erythrocyte membranes, to the identification of these moieties as amphiphilic, ring-structured hexamers, and to the concept of pore formation by the membrane-bound toxin molecules (5, 7, 8, 13). This concept states that alpha-toxin damages membranes by forming stable transmembrane pores through insertion of oligomeric aggregates (predominantly hexamers) into the bilayer. Studies conducted on a biochemical level (26) and a functional level (2a, 20, 24, 25) yielded data in line with this concept.

Nevertheless, several aspects of toxin-membrane interaction remain unresolved. For example, rabbit erythrocytes are approximately 100 times more susceptible to hemolytic toxin action than human erythrocytes (3, 19). Earlier data obtained with the use of radioiodinated toxin indicated that this might be due to the presence of high-affinity binding sites (receptors) on rabbit cells (10). In this case, membrane damage could conceivably occur through a mechanism distinct from pore formation (14). In fact, technical limitations

have allowed detection of toxin oligomers only on target membranes treated with relatively high toxin concentrations (10^{-7} M), and quantitative data on the binding characteristics of alpha-toxin to cells are sparse. Such studies have been impeded by the fact that radiolabeling of the toxin to high specific activity tends to destroy its cytolytic activity. Recently, toxin binding was estimated by a hemolytic titration assay, and it was found that toxin binding to rabbit erythrocytes was very ineffective in concentration ranges of 1 to 10 $\mu\text{g/ml}$, but binding increased to higher levels when the toxin concentration was raised to 100 $\mu\text{g/ml}$ (6). Comparable data were obtained by Phimister and Freer (22), who used radioiodinated, active toxin in their study. Both sets of data were difficult to reconcile with a receptor-mediated mode of toxin action on rabbit cells.

Future studies on toxin-membrane interactions would be greatly aided if a means for distinguishing and separately quantitating the mono- and oligomeric forms of the toxin on a membrane could be devised. In this communication, we report the development of a highly sensitive sandwich enzyme-linked immunosorbent assay (ELISA) based on the use of monoclonal and polyclonal antibodies. We show that this ELISA allows simultaneous quantitation of mono- and oligomeric alpha-toxin and that the monoclonal antibody neutralizes the toxin by preventing its binding to both rabbit and human erythrocytes. In an accompanying report (23), we characterize toxin binding to rabbit erythrocytes, study the formation of oligomers at low and elevated temperature, and show that the process of membrane damage is invariably linked to that of oligomer formation.

MATERIALS AND METHODS

Staphylococcal alpha-toxin was obtained as lyophilized material containing approximately 80% (by weight) 3S native

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toxin from D. K. Hungerer (Behringwerke, Marburg, Federal Republic of Germany). Ten milligrams of this material was chromatographed over a Sephacryl S-300 column equilibrated in 20 mM Tris–100 mM NaCl (pH 8.0) as described previously (5), and the 3S native toxin peak was stored in samples of 1 ml of -20°C . Protein was determined by the Lowry et al. method (18). A single major protein band of M_r 34,000 was observed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (13).

Production of monoclonal antibodies. Murine monoclonal antibodies were generated as previously described (15) by using trypsinized alpha-toxin as antigen. A solution containing 1 mg of toxin per ml was trypsinized at a final enzyme concentration of 40 $\mu\text{g/ml}$ for 8 h at 22°C , and the reaction stopped with trypsin inhibitor (40 $\mu\text{g/ml}$; E. Merck AG, Darmstadt, Federal Republic of Germany). The ensuing inactivation of alpha-toxin was checked by hemolytic titration and SDS-PAGE (17). BALB/c mice were immunized by one subcutaneous and one footpad injection of 0.5 ml of trypsinized toxin in complete Freund adjuvant. Four weeks later, a booster injection of 0.5 ml of antigen in incomplete Freund adjuvant was applied subcutaneously. Final immunizations were performed 3 to 5 weeks thereafter with 50 μg of antigen applied intraperitoneally and intravenously without adjuvant 4, 3, and 2 days before fusion of the cells.

Myeloma cells (cell line X63-Ag8.6.5.3) were fused with spleen cells derived from immunized mice by using 50% polyethylene glycol as the fusing agent (15). Hybridoma supernatants were screened for reactivity by ELISA with alpha-toxin absorbed onto Nunc 96-well plates (Nunc, Wiesbaden, Federal Republic of Germany) and by Western blot analyses. Positive hybridoma cells were cloned three times by limiting dilution. Large-scale production and purification of monoclonal antibodies were performed as described previously (15).

Preparation of samples for ELISA. Heat-aggregated alpha-toxin was prepared by incubating toxin solutions (100 $\mu\text{g/ml}$ in phosphate-buffered saline) in a water bath at 60°C for 120 min. Native and heat-aggregated toxin samples (50 to 100 $\mu\text{g/ml}$) were analyzed in triplicate; i.e., (i) toxin without detergent; (ii) toxin treated with 1% (vol/vol) Triton X-100; and (iii) toxin treated with 0.5% (wt/vol) SDS for 1 min at 100°C . All samples were subsequently diluted 1:100 or 1:200 in blocking buffer (20 mM Tris, 150 mM NaCl, 0.3% Tween 20, pH 7.5), and then serial double dilutions were prepared in the same buffer for application in the ELISA.

Toxin-treated rabbit erythrocyte membranes were prepared by incubating 1 ml of washed erythrocytes (10^9 cells per ml) with toxin at final concentrations of 1 or 10 $\mu\text{g/ml}$ for 45 min at 37°C . Membranes were pelleted in a tabletop Eppendorf centrifuge and washed four times with ice-cold 5 mM phosphate buffer (pH 8.0). The membranes were solubilized either with 1% (vol/vol) Triton X-100 at 22°C or with 0.5% (wt/vol) SDS for 1 min at 100°C (both detergents from Serva, Heidelberg, Federal Republic of Germany) and similarly diluted in blocking buffer.

Liposomes were prepared as follows. Phosphatidylcholine (10 mg, type V-E; Sigma, Munich, Federal Republic of Germany) in 0.1 ml of chloroform and 2 mg of cholesterol (Merck) dissolved in 0.2 ml of chloroform were mixed and evacuated to dryness. The lipid mixture was solubilized in 0.8 ml of 100 mM *n*-octylglucoside (Boehringer GmbH, Mannheim, Federal Republic of Germany)–20 mM Tris hydrochloride–150 mM NaCl (pH 8.0), and the clear lipid-detergent solution was dialyzed against 1000 ml of detergent-

free buffer (20 mM Tris, 150 mM NaCl, pH 8.0) at room temperature for 24 h. The resulting liposome suspension (0.8 ml) received 0.2 ml of alpha-toxin (0.5 mg/ml of stock solution). After 20 min at 37°C , the liposome-toxin mixture was solubilized in 100 mM octylglucoside, and the sample was chromatographed over a Sephacryl S-300 column (Pharmacia, Uppsala, Sweden; 1- by 60-cm column) equilibrated with 10 mM Tris–50 mM NaCl–2.5 mM Triton X-100 (pH 8.0) at a flow rate of 4 ml/h in a cold room (4°C). Fractions of 1.3 ml were collected. Samples were subjected to SDS-PAGE-immunoblotting and analyzed by ELISA. For the latter determinations, either 100- μl samples were treated with 0.5% (wt/vol) SDS, boiled (1 min), and then diluted in 100 volumes of blocking buffer, or 10- μl samples were directly diluted in blocking buffer. The diluted samples were then further doubly diluted in the micro-ELISA plates.

ELISA for alpha-toxin. The purified monoclonal antibody clone $\alpha 4\text{C1}$ was absorbed (2 $\mu\text{g/ml}$ in 0.05 M sodium bicarbonate buffer [pH 9.6], 16 to 22 h at 22°C) onto polystyrene micro-ELISA plates (Nunc) and used to capture the monomeric form of alpha-toxin. Before antigen application, the ELISA plates were washed for 20 min in blocking buffer. Antigen incubation was for 60 min at 37°C . Bound antigen was detected with the use of a polyclonal rabbit antibody against alpha-toxin (6), diluted 1:5,000 in dilution buffer (20 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.5), and incubated for 30 min. This step was followed by a 30-min incubation with biotinylated anti-rabbit immunoglobulin G (Amersham, Braunschweig, Federal Republic of Germany; dilution, 1:1,000). Assays were developed with streptavidin-biotinylated horseradish peroxidase complex (Amersham) diluted 1:1,000 in dilution buffer and incubated for 20 min. The enzyme reaction was performed in citrate buffer (pH 5.0), with *o*-phenylenediamine as the chromogen. The reaction was stopped after 10 to 15 min by the addition of 4 N H_2SO_4 . Absorbance was read at 492 nm in an SLT reader EAR 400 (SLT Lab Instruments, Overath, Federal Republic of Germany). All incubation steps were done at 37°C in a humid box, and four washings with 20 mM Tris–500 mM NaCl–0.05% Tween 20 (pH 7.5) were carried out between each incubation step.

Toxin neutralization assays. Purified monoclonal antibodies were doubly diluted in saline, and 1 volume of toxin was added to a final concentration of 1 $\mu\text{g/ml}$. After 30 min at 22°C , one volume of 2.5% rabbit erythrocytes was added, and hemolysis was read after 60 min at 37°C . An anti-streptolysin O antibody (15) served as a control.

SDS-PAGE and Western blotting. A discontinuous gel system was used as described previously (6). All samples received 4% SDS and were incubated at room temperature for 5 min before electrophoresis. Identical results were obtained irrespective of addition or omission of 5 mM dithiothreitol to the samples. Biotinylated molecular weight markers were purchased from Bio-Rad Laboratories (Munich, Federal Republic of Germany). The marker kit contained lysozyme (M_r , 14,400), trypsin inhibitor (M_r , 21,500), carbonic anhydrase (M_r , 31,000), ovalbumin (M_r , 43,000), bovine serum albumin (M_r , 66,000), and rabbit muscle phosphorylase *b* (M_r , 97,400). Western blotting (immunoblotting) was performed as described previously (6), and the blots were incubated with primary antibodies (dilution, 1:1,000) followed by biotinylated second antibodies (Sigma or Dakopatts, Copenhagen, Denmark; dilution, 1:1,000) and streptavidin-biotinylated horseradish peroxidase complex (Amersham; dilution, 1:1,000). Development was with 3-amino-9-ethylcarbazole as described previously (6).

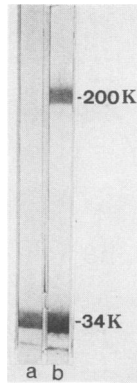


FIG. 1. SDS-PAGE-Western blot of rabbit erythrocyte membranes (20 μ l) lysed with 10 μ g of alpha-toxin per ml. Blots were developed with (a) monoclonal antibody α 4C1 or (b) polyclonal rabbit antibody. Note staining of both 200K and 34K toxin bands in lane b but lack of reactivity of monoclonal antibody α 4C1 with 200K toxin. Polyclonal mouse antibodies yielded the same staining patterns as shown in lane b.

RESULTS

Monoclonal antibody α 4C1 selectively recognizes monomeric alpha-toxin in Western blot analyses. Figure 1 depicts a Western blot analysis of SDS-solubilized rabbit erythrocyte membranes that had been treated with 10 μ g of alpha-toxin

per ml; the samples were not boiled before electrophoresis to preserve some of the hexamers (13). Polyclonal mouse and rabbit antitoxin antibodies stained both of these toxin forms (Fig. 1, lane b) (6). In contrast, the monoclonal antibody α 4C1 recognized only the monomeric form of alpha-toxin (Fig. 1, lane a).

Sandwich ELISA for quantifying mono- and oligomeric alpha-toxin. The monoclonal antibody was adsorbed onto polystyrene plates. Different forms of alpha-toxin were offered as antigens, and the ELISA was developed with polyclonal rabbit antitoxin antibodies and revealed with a streptavidin-biotin peroxidase system.

First, the effects of detergents on native alpha-toxin were examined. As expected, treatment of native alpha-toxin with the nondenaturing detergent Triton X-100 caused no alteration in the ELISA reactivity of the antigen (Fig. 2). The sandwich assay was very sensitive, with a lower detection limit of 0.25 to 0.5 ng of toxin per ml ($\sim 10^{-11}$ M). A toxin sample that had been boiled in 0.5% SDS could not be applied directly to the ELISA because of the denaturing effects of the detergent on the antibody system. However, full antigenic activity was observed when the sample was diluted at least 100-fold (Fig. 2A).

Next, heat-aggregated toxin was analyzed. Heating at 60°C resulted in formation of water-insoluble aggregates with concomitant loss of hemolytic activity (Fig. 2B) (1). Early studies of Arbuthnott et al. showed that such heat treatment caused formation of toxin hexamers (1). The aggregated

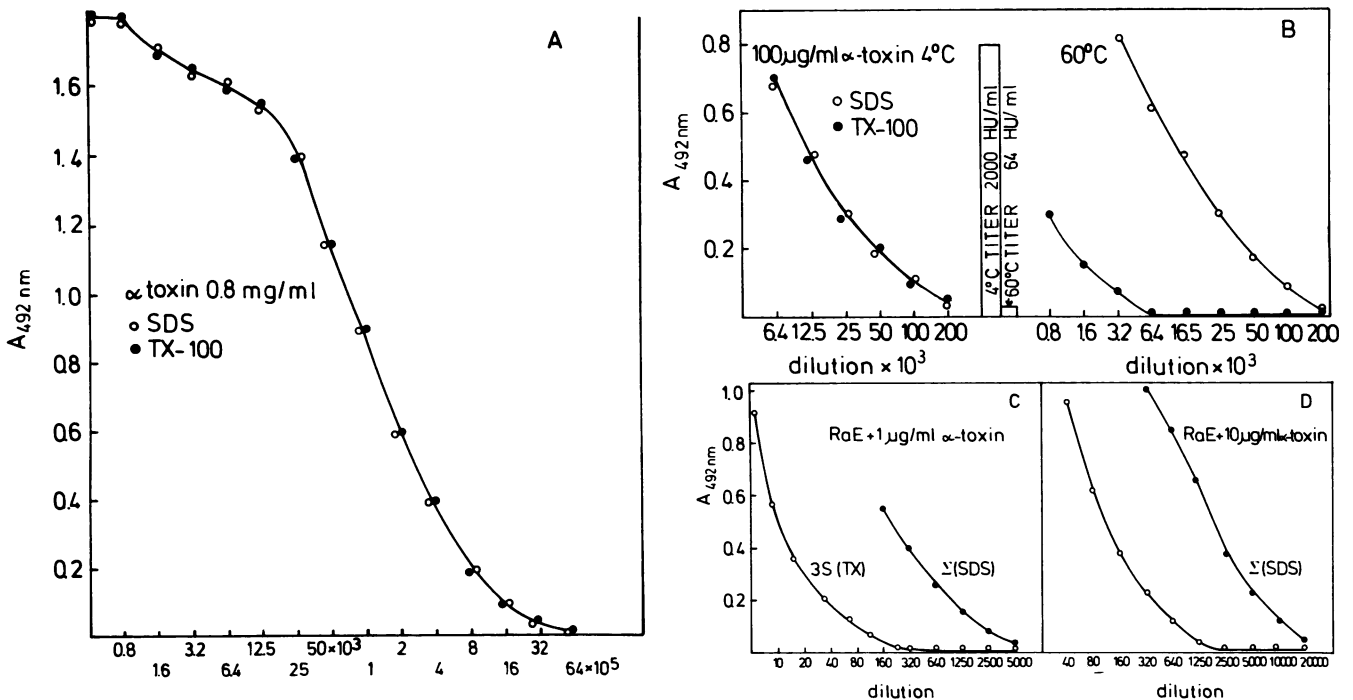


FIG. 2. (A) ELISA calibration curve obtained with alpha-toxin after treatment with 1% Triton X-100 or 0.5% SDS. The same curve was obtained with native toxin. Note the full antigenic renaturation of SDS-treated toxin after dilution (≥ 100 -fold) in blocking buffer. (B) A solution (100 μ g/ml) of alpha-toxin was incubated at 4°C (control) or 60°C for 120 min. The control toxin exhibited a hemolytic activity of 2,000 hemolytic units (HU) per ml and identical Triton X-100 (TX-100) versus SDS ELISA reactivity (left curve). After heating, toxin activity was reduced to 64 hemolytic units per ml, and a corresponding decrease in Triton X-100-reactive material (native 3S toxin) was observed. SDS treatment liberated 3S toxin from the aggregates, resulting in normalization of ELISA values (O, right curve). (C and D) ELISA of rabbit erythrocyte membranes after treatment with 1 (C) or 10 (D) μ g of alpha-toxin per ml. SDS solubilization yielded data for total bound toxin (●), whereas monomeric toxin was quantitated by solubilization in Triton X-100 (O). The difference between SDS and Triton X-100 values represented toxin oligomers.

toxin preparation could be dissolved by incubation with 1% Triton X-100 or by boiling in 0.5% SDS. The Triton-treated material was, however, reactive only to a minor extent in the ELISA, and this residual antigenic activity correlated exactly with the extent of residual hemolytic activity of the toxin solution. In contrast, antigenic reactivity was fully restored after boiling in SDS (Fig. 2B). These results further indicated that the ELISA detected native, but not oligomerized, toxin.

Finally, rabbit erythrocyte membranes that had been lysed with alpha-toxin were analyzed. Cell suspensions (10^9 cells per ml) were treated with 1 or 10 μg of toxin per ml for 45 min at 37°C. The washed membranes were then solubilized with either Triton X-100 for quantitation of bound monomers or with SDS for quantitation of total bound toxin. Membranes treated with 1 μg of toxin per ml were found to carry a total of ~1,000 ng of toxin per ml (Fig. 2C). Since the packed membrane pellets contained approximately 10^{10} ghosts per ml, this corresponded to an average of approximately 2,000 toxin molecules per cell. Of these, however, only a minority (~7%) represented monomers, as evident from analysis of Triton X-100-solubilized membranes (Fig. 2C). The difference between total and monomeric toxin could only represent nonreactive toxin oligomers. Figure 2D depicts an ELISA of membranes that had been lysed with 10 μg of toxin per ml. The total toxin concentration on membranes was now approximately 7 $\mu\text{g}/\text{ml}$ or ~12,000 molecules per cell, and monomer toxin comprised only about 8% of total bound toxin. Thus, it is evident that the mass of toxin molecules bound to target membranes at 37°C is in oligomeric form. The sensitivity limit for Triton X-100-solubilized membranes is in the range of 0.5 ng of monomeric toxin per ml, corresponding to approximately 10^{10} toxin molecules per ml or an average of only 1 toxin molecule per cell. The detection limit for SDS-solubilized membranes is approximately 100-fold higher (100 toxin molecules per cell) because of the necessity of diluting the samples 100-fold before analysis.

Analyses of alpha-toxin after oligomerization on liposomes. In these experiments, toxin oligomerization was induced through incubation with protein-free lipid vesicles composed of phosphatidylcholine and cholesterol. Earlier studies (2) established that this procedure leads to formation of toxin oligomers. Samples were resolubilized with nondenaturing detergent and chromatographed over Sephacryl S-300; this step has previously been shown to separate monomeric toxin (M_r , 34,000; 34K toxin) and oligomeric (200K) toxin (5). The column fractions were analyzed by ELISA. When samples were boiled in SDS before application in the ELISA plates, two toxin peaks were detected, corresponding to monomeric and oligomeric toxin. The latter peak displayed a small shoulder indicative of the presence of some molecular aggregates that were larger than hexamers (Fig. 3A). When analyzed by SDS-PAGE-immunoblotting with polyclonal antibodies, fractions from the first peak generated the 200K hexameric toxin band in addition to the 34K monomer band, whereas fractions from the rear peak generated the 34K band only (Fig. 3C). However, immunoblots developed with the monoclonal antibody α 4C1 again resulted in the absence of staining of the 200K band (Fig. 3B). These findings directly documented that nondenatured, high-molecular-weight toxin oligomers generated by contact of the toxin with lipid membranes were unreactive in the ELISA, thus corroborat-

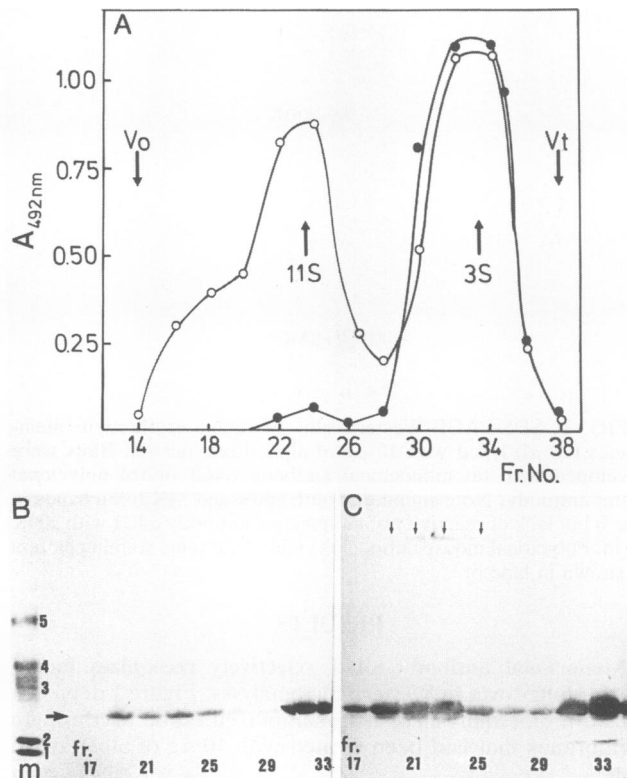


FIG. 3. *S. aureus* alpha-toxin was incubated with phosphatidylcholine liposomes to induce formation of oligomers. The liposome-toxin mixture was subsequently solubilized with octylglucoside and chromatographed over Sephacryl S-300 in the presence of Triton X-100. (A) Analyses of column fractions by ELISA with (○) or without (●) prior treatment with SDS; absorbance values shown are for samples diluted 1:400. Vo, Void column volume; Vt, bed volume. The elution positions of monomeric 3S and hexameric 11S toxin are indicated. (B) Analysis of column fractions by SDS-PAGE followed by Western blotting with monoclonal α 4C1 antibodies; lane m was loaded with the following biotinylated marker proteins: 1, lysozyme and trypsin inhibitor (not resolved in this gel system); 2, carbonic anhydrase (M_r , 31,000); 3, ovalbumin (M_r , 43,000); 4, bovine serum albumin (M_r , 66,000); 5, rabbit muscle phosphorylase b (M_r , 97,400). The arrow denotes the position of monomeric toxin (M_r , 34,000). (C) Parallel analysis with polyclonal rabbit antibodies, showing the additional staining of hexameric toxin (M_r , 200,000; arrows) that is residually present in the high-molecular-weight fractions from the column.

ing the contention that the monoclonal antibody reacted solely with the monomeric toxin form. Since the liposomes were devoid of protein, these experiments also showed that nonreactivity of the oligomers was not due to concealment of the reactive epitope by membrane proteins.

Monoclonal antibody α 4C1 neutralizes alpha-toxin by preventing its binding to both rabbit and human erythrocytes. Monoclonal antibody clone α 4C1 possessed toxin-neutralizing properties. In the experiment depicted in Fig. 4, a solution of 250 μg of purified monoclonal antibody per ml was doubly diluted, and toxin was added to a final concentration of 1 $\mu\text{g}/\text{ml}$ to each well. After 30 min, rabbit erythrocytes were added, and ensuing hemolysis was registered. A monoclonal antibody to streptolysin O was analyzed in parallel as a negative control. Specific neutralization of alpha-toxin by monoclonal antibody α 4C1 was observed, and 1 μg of toxin per ml was neutralized by 2 μg of antibody

per ml. These results indicated that one antibody molecule was capable of neutralizing two molecules of alpha-toxin.

The ELISA was then used to determine whether neutralization was due to inhibition of toxin binding to cells. When toxin was incubated with the antibody in solution, it could not be detected in the ELISA because the reactive epitope was already bound to antibody (data not shown). However, boiling of the sample in SDS irreversibly destroyed the antigen-binding site on the monoclonal antibody, releasing the toxin and rendering it available for reaction in the ELISA (Fig. 5, bars a). Therefore, if the monoclonal antibody did not inhibit toxin binding to membranes, the toxin would be released from immune complexes by boiling the membranes in SDS and become detectable in the immunoassay.

Solutions of 10 and 20 μg of alpha-toxin per ml were preincubated with neutralizing antibody (100 $\mu\text{g}/\text{ml}$), and human or rabbit erythrocytes were added. The unlysed cells were washed and lysed hypotonically, and the membranes were analyzed for bound toxin. Preincubation of toxin with the monoclonal antibody resulted in virtual absence of toxin binding to either rabbit or human erythrocytes (Fig. 5, bars b through d).

Similar results were obtained when toxin was preincubated with antibody at 0°C , followed by incubation with erythrocytes at 0°C for 60 min. Again, no toxin became cell bound under these conditions.

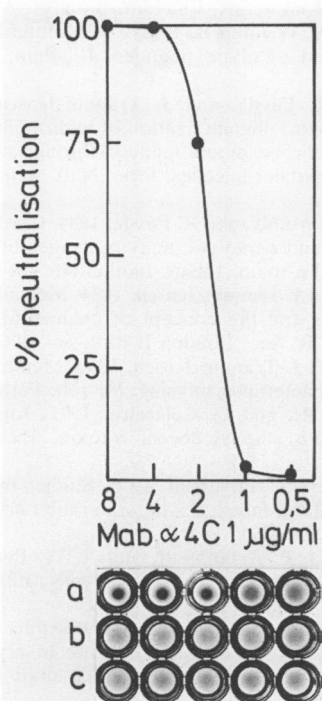


FIG. 4. Neutralization of alpha-toxin hemolytic activity by monoclonal antibody clone $\alpha 4C1$. Samples (50 μl) of a purified immunoglobulin G preparation were doubly diluted, and then alpha-toxin was added to a final concentration of 1 $\mu\text{g}/\text{ml}$. The final concentration of monoclonal antibody is shown in the curve. After preincubation with the antibodies for 30 min, erythrocytes were added, and the ensuing lysis was recorded. The reciprocal values of percent hemolysis were taken as percent neutralization. The lower panels show assays performed in parallel with a nonrelated monoclonal antibody (row b) and a monoclonal antibody to streptolysin O (row c). Row a is the test performed with anti-alpha-toxin antibody clone $\alpha 4C1$.

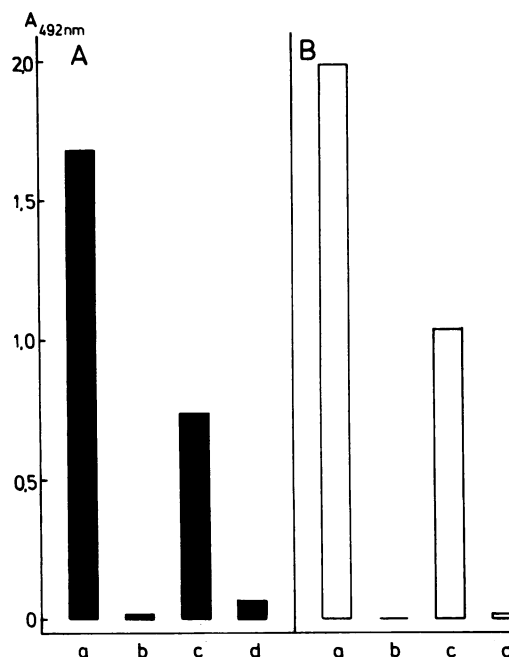


FIG. 5. Monoclonal antibody inhibits toxin binding to cells. For bars a, 10 μg of alpha-toxin per ml was incubated with 20 μg of antibody clone $\alpha 4C1$ per ml for 30 min, 22°C . Samples were then boiled to 0.5% SDS, diluted 100-fold in blocking buffer, and applied to the ELISA. The toxin was released from the immune complexes by boiling in SDS and could be detected subsequently. Other bars: b, ELISA of control rabbit (A) and human (B) erythrocyte membranes without toxin treatment; c, ELISA of rabbit (A) and human (B) erythrocyte membranes after treatment with 10 μg of alpha-toxin per ml; d, ELISA of SDS-solubilized rabbit (A) and human (B) erythrocyte membranes after incubation of cells with 10 μg of alpha-toxin per ml in the presence of neutralizing monoclonal antibody $\alpha 4C1$ (20 $\mu\text{g}/\text{ml}$). The monoclonal antibody inhibited binding of alpha-toxin to both rabbit and human cells.

DISCUSSION

The monoclonal antibody against alpha-toxin described herein has permitted the construction of a sensitive ELISA with the unique capacity to distinguish between mono- and oligomeric forms of membrane-bound toxin. Monomeric toxin, whether in native form or after its liberation from oligomers by SDS treatment, reacts in the ELISA. By contrast, oligomeric toxin reacts neither in Western blots nor in the ELISA. This conclusion is based on three mutually consistent findings. First, the monoclonal antibody reacts with monomeric toxin in Western blots but does not stain the high-molecular-weight 200K toxin band corresponding to SDS-resistant hexamers. This observation was made with toxin that had oligomerized on erythrocyte membranes as well as with toxin oligomerized through contact with protein-free liposomes. Second, the antibody failed to react with heat-aggregated toxin in the ELISA but did react after monomerization with SDS. Third, the antibody also did not react in ELISA with nondenatured toxin oligomers solubilized from liposomes; again, reactivity was observed after conversion of oligomeric toxin to the monomer form in SDS. The liposome experiments excluded the possibility that nonreactivity in the ELISA was due to concealment of the reactive epitope by bound membrane proteins. Failure of the hexamer to react was also unlikely to be due to the binding of detergent; SDS-PAGE extensively removes both lipid and

detergent from proteins, yet the oligomeric toxin band was still not stained by the monoclonal antibody. In other experiments (data not shown), we also found that addition of Triton X-100 extracts of erythrocyte membranes to alpha-toxin solutions did not affect the reactivity of the monomer in the ELISA.

By comparing the total concentration of membrane-bound toxin (assayed after SDS solubilization) versus the concentration of toxin monomers (assayed by solubilization in Triton X-100), the concentration of oligomeric toxin can be determined. A decisive advantage of the immunoassay is that toxin is used in its active form, and detailed binding studies like those reported in the accompanying paper (23) do not require the preparation of radiolabeled protein. The ELISA is very sensitive, due probably to a high affinity of the monoclonal antibody for the toxin, and can detect an average of 1 molecule of monomeric toxin and 100 molecules of oligomeric toxin (16 hexamers) per erythrocyte. Results obtained with the ELISA have demonstrated that binding of alpha-toxin to target membranes at 37°C indeed results in formation of oligomers, monomeric toxin being present in minor amounts only.

It was of interest to determine the mechanism of toxin neutralization by monoclonal antibody α 4C1. Neutralization could conceivably occur through two distinct mechanisms, i.e., primary inhibition of toxin binding, or inhibition of oligomerization and pore formation by bound toxin. The latter mechanism has been shown to be operative in the case of a neutralizing antibody against streptolysin O (15), which did not block toxin binding to erythrocytes but inhibited oligomerization and pore formation of cell-bound toxin. In contrast, the present antibody to alpha-toxin appears to act by primarily inhibiting toxin binding to cells. Incubation of toxin with monoclonal antibody prevented toxin binding to both human and rabbit erythrocytes. This finding alone would not exclude the possibility that the antibody primarily blocked oligomerization, which in turn might be a prerequisite for firm binding of the toxin to membranes. However, as reported in the accompanying communication (23), incubation of rabbit erythrocytes with low levels of toxin at 0°C results in firm binding of monomers without oligomer formation, and this binding is also blocked by the monoclonal antibody. Furthermore, when monomers are first allowed to bind to cells at 0°C, they are no longer neutralized by subsequently added antibody (23). These findings collectively argue for true primary inhibition of toxin binding in its monomer form to membranes by monoclonal antibody α 4C1. Since the antibody cannot react with oligomerized toxin, we conclude that the relevant epitope becomes concealed due to a conformational change accompanying oligomerization and, presumably, bilayer penetration. The stage of oligomerization at which the epitope disappears is unknown and may, for example, occur already at the level of dimer formation. Since oligomers of defined size other than hexamers cannot be generated and isolated at present, this issue cannot yet be resolved. At this stage, we use the term "oligomer" to denote all forms of nonreactive toxin.

The collective results presented in this communication suggest that monoclonal antibody α 4C1 reacts with a molecular domain in the toxin molecule that is spatially close to or even identical with its membrane-binding site. Since the antibody blocks binding of toxin to both rabbit and human erythrocytes, it follows that the same site is involved in binding to both cell species. If correct, this contention is difficult to reconcile with the reported existence of specific membrane receptors on rabbit cells for alpha-toxin (10). In

the accompanying report, the process of membrane binding and toxin oligomerization in rabbit erythrocyte membranes is discussed in detail, and the results also speak against the existence of cell surface receptors for alpha-toxin on rabbit erythrocytes (23). While this paper was under review, a report appeared that alpha-toxin may be binding to the choline head group of phosphatidylcholine (27). If monoclonal antibody α 4C1 is directed against this choline recognition domain, it may aid in future identification of the peptide sequence(s) that is involved in this unique interaction.

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