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A Macromolecular Structure Produced by *Pseudomonas aeruginosa* Is Recognized by Antibody to Exotoxin A

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Organized particulate structures (rods) identified in purified preparations of exotoxin A from culture supernatants of *Pseudomonas aeruginosa* PA103 were found to be immunochemicallv cross-reactive with exotoxin A. The rods were visualized by electron microscopy after negative staining as hollow tubes or sheaths (45 by 15 nm). Purified rods were not toxic and not enzymatically active in the ADP-ribosylation assay. Antigenic cross-reactivity between exotoxin A and rods was demonstrated by using monoclonal antibodies directed against either rods or a toxoid of exotoxin A. Hybridoma clones derived from mice immunized with rods or toxoid reacted with both antigens in the enzyme-linked immunosorbent assay. Rods could be dissociated by boiling and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis into three subunit polypeptides with molecular weights of 70,000, 45,000, and 27,000. Two of the three subunit polypeptides reacted both with antirod and antitoxin monoclonal antibodies after electrophoretic transfer of sodium dodecyl sulfate–polyacrylamide gel electrophoresis-separated proteins to nitrocellulose filters. The results indicate that rods and exotoxin A share common antigenic determinants.

In this report, we describe an organized, rod-shaped macromolecular structure that was originally observed to copurify with exotoxin A from culture supernatants of *Pseudomonas aeruginosa*. Subsequently, this particulate structure was found to contain common antigenic determinants with exotoxin A. These structures are referred to as rods. Exotoxin A is produced by *P. aeruginosa* as a monomeric proenzyme with a molecular weight of 70,000. The processed form of exotoxin A catalyzes the transfer of ADP-ribose from NAD to eucaryotic elongation factor 2 and inhibits protein synthesis (8). The proenzyme form can be activated by freezing and thawing (22) or by treatment with urea and dithiothreitol (14). Active fragments with molecular weights of 48,000 and 27,000 (48K and 27K fragments) have been generated from the toxin by limited proteolysis (3, 16, 19). The low-molecular-weight fragments are not toxic. In addition, a presumptive precursor form with a molecular weight greater than 90,000 was recently described (P. B. Fernandes and K. R. Cundy, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, B92, p. 32). Thus, there are various molecular forms of the toxin. The nature of the processing events and the relationship(s) between the different molecular species and the native molecule are not known. In addition to exotoxin A, *P. aeruginosa* also produces a variety of extracellular products, including rods. Culture supernatants contain a number of particulate structures, e.g., R-type pyocins and bacteriophage. Several possible mechanisms might explain the incorporation of toxin antigenic determinants into a macromolecular structure. Experiments in this communication are directed toward identifying possible mechanisms that would result in the observed antigenic cross-reactivity.

MATERIALS AND METHODS

Bacterial strains and growth procedures. *P. aeruginosa* PA103 was obtained from P. V. Liu (University of Louisville, Health Science Center, Louisville, Ky.). Bacterial cultures were grown at 32°C in the tryptic soy dialysate medium described by Liu (15) for the production of exotoxin A (BBL Microbiology Systems, Cockeysville, Md.). Bacteria were grown with vigorous shaking at 32°C in 4-liter Erlenmeyer flasks containing 300 ml of media inoculated with 0.2 ml of an 18-h culture. Unless otherwise indicated, mitomycin c (Sigma Chemical Co., St. Louis, Mo.) was added to 12-h cultures to a final concentration of 0.13 mg/ml. The cultures were incubated under the same conditions for 10 h after the addition of mitomycin. The viability of the culture was decreased by one log by the mitomycin treatment, but overt lysis of the culture did not occur. Supernatant fractions from cultures treated with mitomycin yielded a 4- to 10-fold increase in the total protein retained by antibody affinity columns (exotoxin A plus rods) compared with supernatants from untreated cultures (data to be presented elsewhere).

Purification of exotoxin A and macromolecular structures. Exotoxin A was purified from culture supernatants by affinity chromatography essentially as described by Taylor and Pollack (21). Sepharose 4B was activated with cyanogen bromide and reacted with antitoxin immunoglobulin G (IgG) in the ratio of 5 mg of IgG per ml of Sepharose. The crude culture supernatant fraction was concentrated and applied to the column (1.6 by 25 cm) in 0.05 M sodium phosphate (ph 7.0). The retained protein was eluted with 3 M sodium thiocyanate (NaSCN) and dialyzed against four changes (4 liters each) of 0.01 M Tris-hydrochloride (pH 8.0). After affinity chromatography, the antigenically reactive fractions were resolved by gel filtration on a Sephadex G-100 column in 0.01 M Tris-hydrochloride (pH 8.0) to separate exotoxin A and rods.

ADP-ribosyltransferase assay. ADP ribosylation activity of exotoxin A preparations was measured as described by Leppla (14), using elongation factor 2 prepared from rabbit reticulocytes according to Allen and Schweet (1) and [^{14}C]NAD (Amersham Corp., Arlington Heights, Ill). Trichloroacetic acid-precipitated, ADP-ribosylated elongation factor 2 was counted in a Packard scintillation spectrometer.

Activation of enzymatic activity by preincubation with

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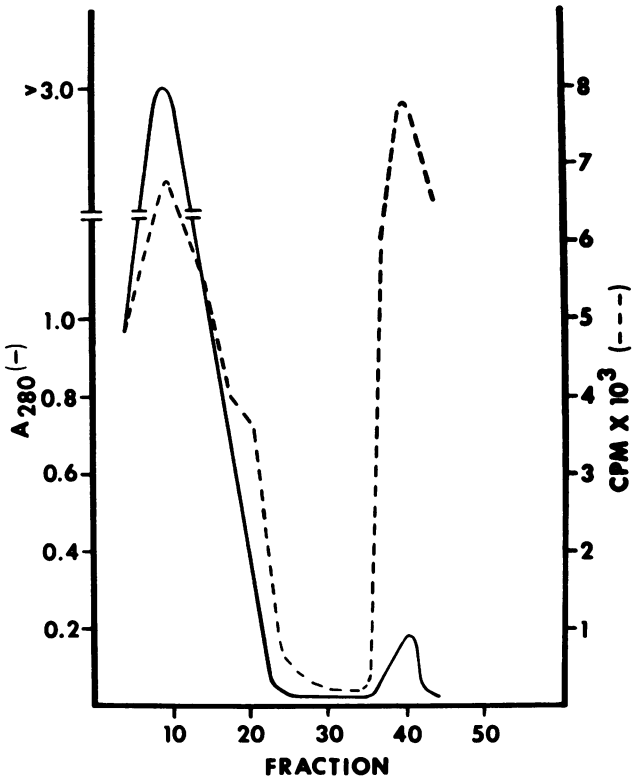

FIG. 1. RIA analysis of the Sephadex G-100 eluate obtained when exotoxin A was purified by the procedure of Liu (15). Fractions 1 to 20 represent the void volume of the column. Fractions 36 to 42 contain purified exotoxin A. The eluate was assayed with rabbit anti-exotoxin A antibody and ^{125}I-labeled protein A in a solid-phase RIA. Microassay plates were coated with eluted protein, washed, and incubated with antibody. The antigen-antibody complex was detected by binding ^{125}I-labeled protein A.

urea and dithiothreitol (15 min, 25°C) was performed as described by Leppla (14). Assays were performed for 15 min, and enzyme activity was calculated as picomoles of ADP-ribose transferred per hour. Protein was estimated from the extinction at 280 and 260 nm and also by the Coomassie blue method of Bradford (2), using ovalbumin as a standard.

Pyocin purification. R-type pyocin and pyocin sheath were isolated from *P. aeruginosa* PA103 as described by Yui (24).

Gel electrophoresis. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) and β-mercaptoethanol was performed as described by Laemmli (13). Samples were boiled for 5 min before electrophoresis. The protein bands were resolved electrophoretically and transferred to nitrocellulose according to Symington et al. (20) to investigate the antigenic relationships of various polypeptides. The nitrocellulose replica was saturated with bovine serum albumin, and the filters were exposed to solutions containing monoclonal antitoxin or monoclonal antirod antibody. The solutions contained equal volumes of phosphate-buffered saline (PBS) and culture media from hybridomas grown as described below. Supernatants from parental myeloma cells diluted 1:5 with PBS served as the negative control. Antibody binding was observed by overnight incubation at room temperature with 5 μCi of ^{125}I-labeled $F(ab^1)_2$ fragment of sheep antimouse immunoglobulin (Amersham) per 144 cm^2 of filter.

Preparation of polyclonal antisera. New Zealand white rabbits (2.5 to 3 kg) were immunized by subcutaneous injection with 10 μg of exotoxin A in complete Freund adjuvant. After 3 weeks, the rabbits were boosted by intramuscular injection of 25 μg of exotoxin A in complete Freund adjuvant. IgG was purified from whole antiserum by precipitation with ammonium sulfate at 40% saturation and then chromatographed on DEAE. The ammonium sulfate precipitate was suspended in 0.85% NaCl, dialyzed against 0.015 M phosphate (pH 8.0), applied to a Whatman DE52 column, and eluted with 0.015 M phosphate (pH 7.75). Optimum antigen-to-antibody ratios for immunoprecipitation and double immunodiffusion assays were determined as previously described (17).

Hybridoma production and screening. Swiss CF-1 mice (20 g, Charles River Breeding Laboratories, Inc., Wilmington, Mass.; Crl:COGS CF-1 BR) were immunized by intraperitoneal injection with either a toxoid of exotoxin A (gift of S. H. Leppla) or the purified macromolecular structure. Splenic lymphocytes were prepared and fused to P3×36-Ag8 myeloma cells essentially according to published protocols (11). Myeloma cells were mixed with splenic lymphocytes at a ratio of 1:5. The cells were incubated overnight in Dulbecco medium containing 20% fetal calf serum, added to HAT medium (hypoxanthine-aminopterin-thymine) to a concentration of 5 × 10^5 cells per ml, dispersed into 96-well

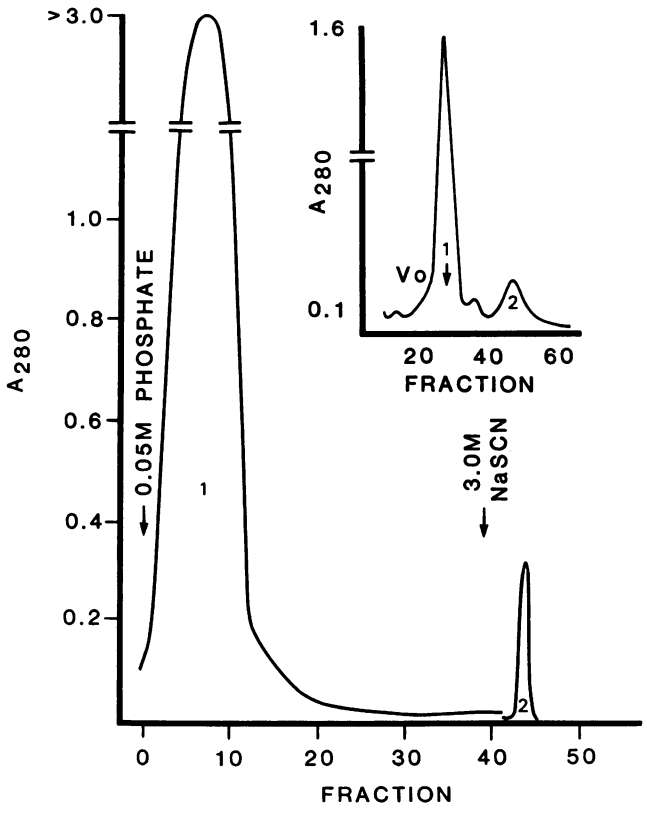

FIG. 2. Elution profiles of exotoxin A. Shown is a typical affinity chromatography profile of crude culture supernatant from PA103. Approximately 700 mg of protein was applied to the column, and 5-ml fractions were collected. Inset: Sephadex G-100 gel filtration of affinity chromatography peak 2. The column was eluted with 0.01 M Tris-hydrochloride (pH 8). The sample was resolved into two protein peaks, one included by the column (peak 2) and one excluded (peak 1).

TABLE 1. Purification data based on enzymatic activity of exotoxin A

Purification stage[a]	Total protein (mg)	Sp act (U/mg)[b]	% Recovery units	LD_{50}[c] (μg)	$TCLD_{50}$[d] (ng)
Crude supernatant	1,430	12.88		3.6	ND
AC peak 1	1,350	6.5	47.8	ND	ND
AC peak 2	3.18	2,680	55.4	ND	ND
G-100 peak 1	2.92	170	5.8	>20	>100
G-100 peak 2	0.67	2,380	18.7	0.5	2.5

[a] Two-liter cultures of PA103 were grown, mitomycin c was added, and the culture supernatants were purified as described in the text. AC, Affinity chromatography.
[b] Measured in the ADP-ribosyl transferase assay as described in the text. One unit = 1 picomole of ADP transferred per hour.
[c] LD_{50}, 50% lethal dose.
[d] Cytotoxicity assays were provided by J. Klinger and were done with cultured L929 cells.

microassay plates, and incubated at 37°C. The resultant clones were screened in an enzyme-linked immunosorbent assay (ELISA). Selected hybridomas were subcloned by the limiting dilution method.

ELISA. A 100-μl amount of either exotoxin A or the macromolecular structure (10 μg/ml) was placed in each well of a 96-well acetate microassay plate (Linbro Scientific, Inc., Hamden, Conn.) and incubated overnight in a humidified atmosphere. The wells were washed three times with wash buffer (0.25 ml of Tween–500 ml of PBS). A filler layer of 175 μl of 5% bovine serum albumin in PBS was added, and the plate was incubated at 37°C for 1 h. The wells were washed three times with wash buffer, 80 μl of each hybridoma culture fluid was added to duplicate wells, and the plate was incubated for 3 h at 37°C. The wells were washed three times with wash buffer, 70 μl of alkaline phosphatase-conjugated rabbit antimouse serum was added to each well, and the plate was incubated overnight at room temperature. The wells were washed four times with wash buffer, and 60 μl of *p*-nitrophenylphosphate (1 mg/ml in 0.05 M Na_2CO_3–0.001 M $MgCl_2$, pH 9.8) was added to each well. After 30 min, the reaction was stopped by the addition of 60 μl of 3 N NaOH. Qualitative results were read by placing the plate against a white card and observing a yellow color change. For quantitation, the absorbance at 405 nm was read spectrophotometrically with a Bio-Tek Chromoscan microplate reader (Bio-Tek Instruments Inc., Shelburne, Vt.). Competition ELISAs were performed in essentially the same manner, except that the monoclonal antibody solution was preincubated for 3 h at 37°C with an equal volume of rod or toxin solution (diluted in PBS to the concentration indicated in the figures). Controls containing no competitor were diluted 1:1 with PBS and incubated at 37°C for 3 h.

Mouse lethality. Toxicity for mice was determined by intraperitoneal injection of 0.5 ml of the indicated exotoxin A or rod preparation into 20-g Swiss CF-1 mice (Charles River). Mice were observed for 5 days. The 50% lethal dose of a given preparation was determined from the dose-response curve as described by Wilson and Miles (23).

Electron microscopy. Samples were prepared for electron microscopy in the following manner: aliquots were spotted onto a clean piece of Parafilm (American Can Co., Greenwich, Conn.). A carbon-coated grid was floated film side down on the sample drop, washed by floating on successive water drops, stained by floating for 30 s on a drop of 2% phosphotungstic acid, and washed by floating on successive water drops. Observations were made with a Philips 200 electron microscope.

RESULTS

Identification and purification of an antitoxin-reactive organized rod structure. Two methods of purification of exotoxin A were employed in this study: the method of Liu et al. (15), using protein precipitation and gel filtration, and the method of Taylor and Pollack (21), using antibody affinity chromatography. Each of the purification stages of exotoxin A was routinely monitored by radioimmunoassay (RIA). RIA analysis of the Sephadex G-100 eluate always yielded two peaks of antigenic activity when exotoxin A was purified by the Liu method. One protein peak was excluded and one peak was included by the gel filtration column (Fig. 1). The protein eluting in the included fraction consisted of pure exotoxin A, as judged by enzymatic activity and electrophoretic profile (data not shown). The protein excluded by the Sephadex G-100 column was shown by SDS-polyacrylamide gel electrophoretic analysis to be composed of three major polypeptides and a number of minor constituents, each of which independently would be retained by G-100 gel filtration. The electrophoretic analysis suggested that the high-molecular-weight immunoreactive material represented an aggregate or an associated structure. A purification procedure based on the antibody affinity chromatography method of Taylor and Pollack (21) was adopted to determine which of the several polypeptides detected in the excluded fraction were cross-reactive with exotoxin A. The antibody affinity column provides a specific and direct method of isolating exotoxin A and related proteins from culture supernatant fractions. Concentrated culture supernatant was applied to the affinity column, and unbound protein was washed from the column with phosphate buffer (Fig. 2). The retained protein was eluted from the column in a small asymmetrical peak with 3 M NaSCN. After dialysis against 0.01 M Tris-hydrochloride (pH 8.0), both the retained and nonretained fractions were assayed for enzymatic activity in the ADP ribosylation assay. Table 1 shows purification data based on the enzymatic activity of exotoxin A and includes measurements of toxicity. The majority (approximately 55%) of the applied enzymatic activity was recovered in the retained fraction. The protein retained by the affinity column was resolved by gel filtration on Sephadex G-100 to separate the rods from exotoxin A. This procedure also resolved two fractions, one included and one excluded. Enzymatic activity catalyzing the ADP ribosylation reaction and toxicity for eucaryotic cultured cells or mice were found only in the included peak. By these criteria, the included fraction represents purified exotoxin A. The excluded fraction was not

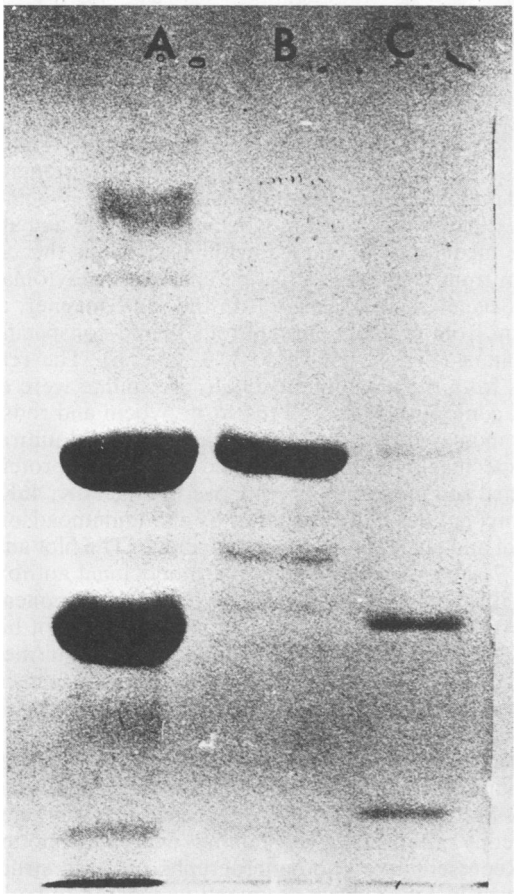

FIG. 3. SDS-polyacrylamide gel electrophoresis of Sephadex G-100 peaks 1 and 2. The two peaks obtained by Sephadex G-100 column chromatography were analyzed by SDS-polyacrylamide gel electrophoresis. The samples were boiled for 5 min before electrophoresis. Lane A, bovine serum albumin (molecular weight, 68,000), ovalbumin (molecular weight, 45,000), and cytochrome *c* (molecular weight, 12,250), molecular weight standards; lane B, Sephadex G-100 peak 2, 45 μg; lane C, Sephadex G-100 peak 1, 18 μg.

enzymatically active or toxic. Both fractions were reactive in the RIA. The majority of the protein applied to the column was recovered in the void volume of the column (the excluded peak). The two Sephadex fractions were analyzed by SDS-polyacrylamide gel electrophoresis, and the exotoxin A fraction contained a 70K protein (Fig. 3). Minor bands could be observed only when the protein load was greater than 40 μg. The excluded fraction contained a mixture of three polypeptides of molecular weights 70,000, 46,000, and 27,000. The 46K and 27K proteins predominated, and the 70K protein was present in smaller amounts. The approximate ratio of the 70K, 46K, and 27K proteins was 0.5:2:1 when estimated by densitometry. The protein eluting in the excluded peak was composed of polypeptides which independently would have been included by the G-100 column, again suggesting that the protein in this fraction was aggregated or in the form of an associated structure.

Immunochemical reactions of exotoxin A and rods. The specificity of the antibody reaction with rod protein was examined, because the original RIA data and the electrophoretic analysis of the affinity-purified Sephadex G-100 eluate suggested the presence of a macromolecular species recognized by antitoxin antibody. Competition RIAs were performed by using antitoxin antibody, exotoxin A as the plate antigen, and increasing concentrations of the macromolecular fraction as a competing antigen. The results of these experiments indicated that there was an antibody population that recognized both exotoxin A and the macromolecule. Ouchterlony double diffusion assays (18) also suggested that exotoxin A and the rods were immunochemically related, because antitoxin reacted with both antigens and formed a spur of partial identity that pointed to the well containing exotoxin A. However, the preceding observations with RIA, immunodiffusion, and affinity chromatography could be explained if the exotoxin A preparation used for the initial immunizations were contaminated with rods yielding antibodies reactive with each of the antigens. The results of the competition RIA experiments could also be explained by nonspecific adsorption or trapping of exotoxin A by unrelated macromolecular structures. A series of experiments with monoclonal antibodies was designed to discriminate between the presence of a cross-reactive species and contamination of the initial immunogen.

Monoclonal antibodies were prepared with either exotoxin A or the purified rod preparation as immunogen. Mice were immunized with a toxoid of exotoxin A or the purified rods, and splenic lymphocytes fused with mouse plasmacytoma cells as described above. Hybridoma clones were screened for the production of antibody to exotoxin A or the macromolecule by using ELISA. Four of 90 clones screened from the exotoxin A fusion were positive for secretion of anti-exotoxin A. Two of the 50 clones from the rod fusion screened for secretion of antirod antibody were positive for antirod production. One clone secreting antitoxin antibody and one clone secreting antirod antibody were subcloned and then propagated in cell culture in large batches for antibody production.

Preliminary ELISA data suggested that the monoclonal antibodies from each clone bound to both exotoxin A and rods. Antigen titration curves were determined for each antigen, and antigen competition was studied in the ELISA to measure the relative ability of each monoclonal antibody to bind to exotoxin A and rods. Monoclonal antitoxin bound exotoxin A and rods to approximately the same extent when titrated against increasing concentrations of antigen (Fig. 4A). Monoclonal antitoxin did not bind exotoxin A with significantly greater affinity than it bound rods. Monoclonal antirod antibody also bound both exotoxin A and rods with similar affinity (Fig. 4B). More antigen was required to achieve critical binding with monoclonal antirod antibody than with the equivalent antitoxin preparation. Parental myeloma culture supernatant and culture fluid from a hybridoma that did not secrete antitoxin or antirod antibody were used as negative controls. Antigen binding by the negative controls was low (A_{405}, 0.1) and was not affected by increasing concentrations of antigen.

Competition ELISAs were performed for both monoclonal antibodies, using toxin or rods as the plate antigen and increasing concentrations of the homologous and heterologous antigen as the competitive inhibitor. The antibodies were assayed for residual ability to bind to antigen after preincubation of the antibody with increasing amounts of competing antigen. The binding of monoclonal antitoxin and monoclonal antirod to both exotoxin A and rods was decreased significantly by preincubation with either antigen. Binding of monoclonal antitoxin to rods (Fig. 5A) or to exotoxin A (Fig. 5B) was inhibited to a similar extent by preincubation with a given concentration of either the ho-

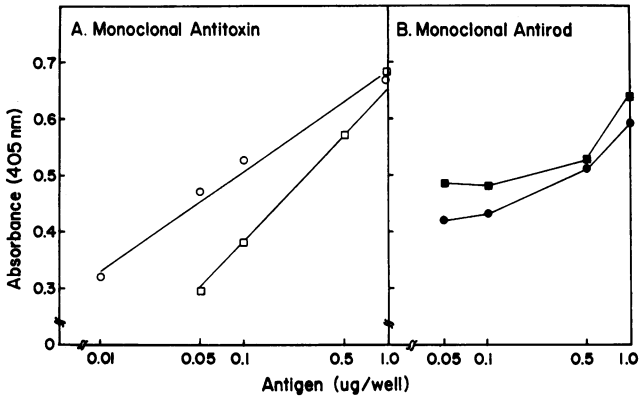

FIG. 4. Antigen specificity of monoclonal antitoxin and antirod antibodies measured by ELISA. Microtiter wells were coated with 100 μl of a solution containing PBS and the amount of exotoxin A (○, ●) or rods (□, ■) indicated. The ELISA reactions were performed as described in the text, using monoclonal antitoxin (A) or monoclonal antirod (B) as antibody.

mologous or heterologous antigen. The same result was obtained when the binding of monoclonal antirod was assayed (Fig. 5C and D). Binding of parental supernatant or the negative hybridoma culture fluid was not influenced by preincubation with either antigen. Rods and exotoxin A share common antigenic determinants (Fig. 4 and 5).

Supernatant fractions of *P. aeruginosa* contain a variety of particulate structures in addition to the rod component. These structures include intact bacteriophages, aeruginosins (pyocins), and related incomplete structures arising from their partial assembly. Antigenic cross-reactivity of rods or any organized structure could result from nonspecific adsorption or accidental inclusion of exotoxin A during assembly. Monoclonal antibodies were used in attempts to distinguish between the possibility of the cross-reactive antigenic determinants residing as an integral part of the structure and their trivial occurrence by adsorption or inclusion. The monoclonal antibodies were employed as probes after protein blotting of SDS-polyacrylamide gels. Previous electrophoretic analysis showed that the macromolecule could be dissociated into three component polypeptides. This experiment was designed to determine whether the cross-reactive determinant(s) reside on the major rod polypeptides.

Several protein preparations were examined in the electroblotting experiments. Purified exotoxin A and partially purified rods (the excluded Sephadex G-100 peak from the Liu purification protocol) were analyzed to detect the presence of common antigenic determinants. R-type pyocin which represents a major particulate component in cultures of strain PA103 was included as a control for the specificity of antibody binding. The polypeptides were electrophoretically transferred to nitrocellulose filters after resolution by SDS-polyacrylamide electrophoresis. The filters were probed with monoclonal antitoxin, monoclonal antirod antibody, or control culture medium from the P3×36-Ag8 plasmacytoma cell line. The immunocomplex was detected by autoradiography as described above. The results are shown in Fig. 6. Specific antibody reaction with exotoxin A was detected by using both monoclonal antibodies, as shown in Fig. 6, panels 2 and 3, Lane A. Two subunits of rods, one with an electrophoretic mobility similar to that of exotoxin A (molecular weight 70,000) and one with an approximate molecular weight of 45,000 reacted specifically with both monoclonal antibodies (Fig. 6, panels 2 and 3, lane B). A third, low-molecular-weight protein also reacted with both monoclonal antibodies. This peptide may represent a degradation product, because it is not usually present in fresh preparations of purified rods.

The R-type pyocin sheath is morphologically similar to rods (6, 7). Electroblotting analysis of pyocin preparations yielded only nonspecific binding reactions. The pyocin polypeptides that reacted with monoclonal antitoxin and antirod culture medium also reacted with material in the culture medium from negative control parental plasmacytoma cells (Fig. 6, panels 1, 2, 3, lane C). In the same manner, a 27K fragment from partially purified rods bound nonspecific cell supernatant (Fig. 6, panels 1, 2, 3, lane B). The reactive species from the ammonium sulfate precipitate were essentially a composite of those present in pyocin and rods. The results indicated that monoclonal antitoxin and antirod reacted specifically only with rod and exotoxin A proteins.

Purified rod preparations composed of the 70K, 46K, and 27K polypeptides were prepared by the immunoadsorption protocol and analyzed as described above. The blot analysis in Fig. 7 shows specific binding of monoclonal antitoxin to both a 70K and a 45K species. The 27K rod component was nonreactive. Parental myeloma supernatant did not bind to any of the rod polypeptides. These results confirmed the observations shown in Fig. 6 and demonstrated that the rod structures contained two structural components that were cross-reactive with exotoxin A.

Properties of the macromolecular structure. Rod preparations purified by immunoaffinity chromatography and Sephadex G-100 gel filtration were examined by electron microscopy after negative staining with phosphotungstic acid. Representative photomicrographs of these structures are shown in Fig. 8. Rods frequently appeared to be joined end-to-end in samples with high concentrations, giving the

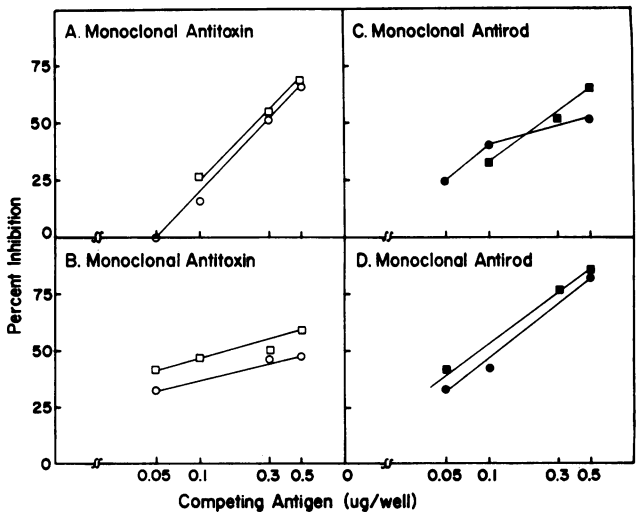

FIG. 5. Competition ELISA. Microtiter wells were coated with 1 μg of exotoxin A (B and D) or rods (A and C). The monoclonal antibodies were preincubated with either exotoxin A (○, ●) or rods (□, ■) at the concentrations indicated and assayed in the ELISA as described in the text. The hydrolysis of *p*-nitrophenylphosphate to product in the absence of competitor was used to define 0% inhibition. The values (A_{405}) for 0% inhibition were: monoclonal antitoxin, toxin on plate, 0.62; rods on plate, 0.66; monoclonal antirod, toxin on plate, 0.73; rods on plate, 0.63. Parental myeloma supernatant and negative hybridoma controls yielded values of A_{405} = 0.24 and A_{405} = 0.23 against toxin and rods, respectively.

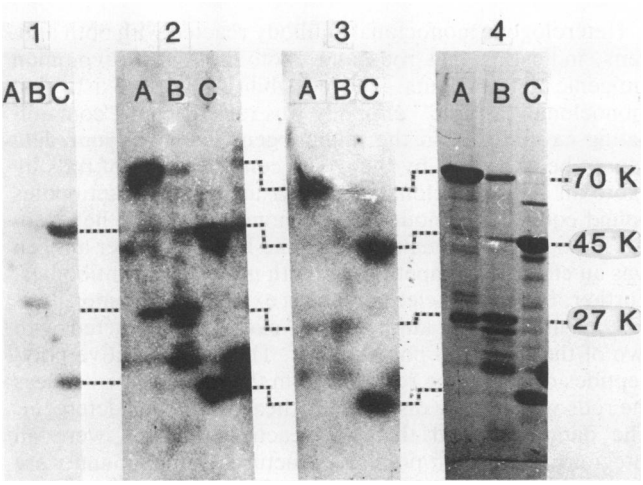

FIG. 6. Analysis of rod proteins with monoclonal antibodies. Exotoxin A, purified according to Leppla (14) (14 μg [A]); rods, G-100 fraction, purified by the method of Liu et al. (15) (7μg, [B]) and pyocin, DEAE fraction (6, 7) (20 μg [C]) were boiled, and the proteins were separated by SDS-polyacrylamide gel electrophoresis. Each sample was loaded on the gel in quadruplicate. One set of samples was stained with Coomassie blue (panel 4), and the other three sets were transferred to nitrocellulose. Panel 1 was probed with control myeloma supernatant; panel 2 was probed with supernatants containing monoclonal antirod antibody, and panel 3 was probed with supernatants containing monoclonal antitoxin antibody. Bound antibody was detected by autoradiography after incubation with ^{125}I-labeled F(ab)$_2$ fragment of sheep antimouse antibody.

appearance of segmented structures (arrows, Fig. 8A). The mean unit length was 45 nm (considering each segment as a unit), and the mean diameter was 15 nm. The rods have the appearance of a hollow tube and accumulate the electron-dense stain in the core section. The rod units resembled the contractile sheath of *Escherichia coli* T-even bacteriophages (12) or the isolated sheath of R-type pyocins (6, 7, 24).

Rods could easily be distinguished from PA103 pyocin-related structures by electron microscopy. The diameter of rods was one-half the diameter of contracted pyocin sheath (15 versus 30 nm) and 1.5 times the diameter of isolated pyocin core. Additionally, fibers or base plate structures were never observed attached to rods. No additional internal detail was apparent in rods, and structural periodicity, ring forms, and protofiliments were not observed. Attempts to visualize altered structures by chemical treatments and derivitization were not successful.

The rod structures were also detected by electron microscopy in purified preparations of exotoxin A prepared by the procedure of Liu (15). Electron microscopic examination of purified exotoxin A prepared by different procedures (gifts of S. Leppla, M. Pollack, and J. Klinger) revealed identical rod structures in all of these samples. Samples of exotoxin A (0.5 mg of protein per ml) that were purified by antibody affinity chromatography followed by Sephadex gel filtration rarely contained any rod structures detectable by electron microscopy. More than 100 rods per 300-mesh electron microscopy grid square were observed in samples of the Sephadex G-100-excluded fraction adjusted to an equivalent concentration (0.5 mg of protein per ml). Thus, affinity chromatography on an anti-toxin IgG Sepharose column followed by gel filtration on Sephadex G-100 is an efficient and convenient method for purifying rods from exotoxin A.

Stability of rods. The most distinctive feature of rods was their stability to physical and chemical treatments that ordinarily dissociate biological structures. The structural integrity of the rods was examined after exposure to a variety of conditions, and the appearance of subunit polypeptides was observed by gel electrophoresis. Intact rod protein did not enter 10% SDS-polyacrylamide gels, and the dissociation of rods could be measured by the appearance of rod polypeptides. Treatment of rods with 5 mM calcium, 500 mM KCl, 100 mM EDTA, 1% SDS, 2% β-mercaptoethanol, 5 M urea, or a combination of SDS, β-mercaptoethanol, and urea failed to dissociate rods or alter their image as seen by electron microscopy. Rods were examined for dissociation after exposure to a number of conditions that dissociate pyocin into subunits because of the morphological similarity of the two structures. Dialysis for 48 h against 8 M guanidine-hydrochloride in 0.1 M phosphate buffer (pH 8.4) or 8 M urea in 0.1 M Na_3PO_4 (pH 12.7) also failed to dissociate rods. Treatment with 10^{-4} M parachloromercuribenzoate did not fully dissociate the structure but did generate what appeared to be shorter rod structures. None of the treatments listed above liberated constituent polypeptides from the rods. All protein remained trapped at the top of the gel when treated samples were analyzed by SDS-polyacrylamide gel electrophoresis. Rod preparations were stable for at least 2 weeks at room temperature or at 37°C, even in the presence of SDS. Boiling in the presence of SDS abolished all structural detail and apparently dissociated rods into constituent polypeptides. All routine samples were boiled before electrophoresis to allow resolution of constituent polypeptides, and this treatment was the only procedure that liberated polypeptides that were cross-reactive with exotoxin A. No exotoxin A enzymatic activity or toxic activity was

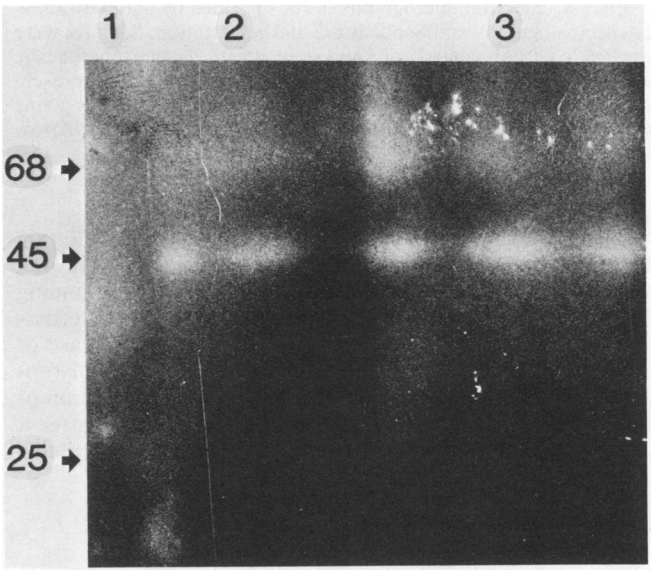

FIG. 7. Monoclonal antibody analysis of rod protein purified by antibody affinity chromatography. Rods (10 μg per well) were loaded in replicate wells, and the rod proteins were separated by SDS-polyacrylamide gel electrophoresis. The proteins were transferred to nitrocellulose and probed with monoclonal antibody. Lane 1, control myeloma supernatant; lane 2, monoclonal antitoxin; lane 3, monoclonal antirod. Molecular weight markers (bovine serum albumin, 68,500; ovalbumin, 45,000; trypsinogen, 25,000) were included in a separate lane and visualized after transfer to nitrocellulose by staining with amido black. The relative mobilities of the marker proteins are indicated by arrows.

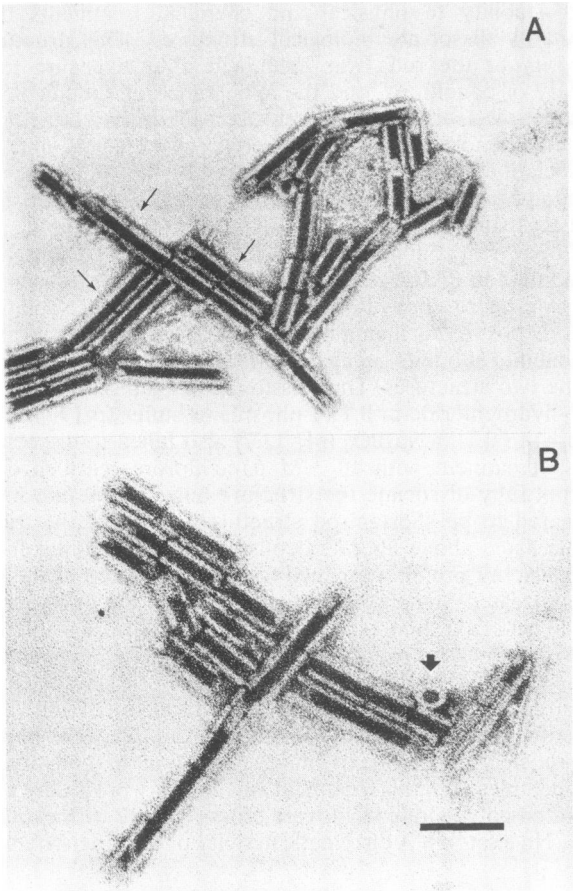

FIG. 8. Electron micrographs of rods purified by antibody affinity chromatography and Sephadex G-100 gel filtration. Samples were applied to carbon-coated grids and stained with 2% phosphotungstic acid. (A) Numerous organized rod-shaped structures were present in the void volume. These structures were frequently joined, giving a segmented appearance, indicated by the arrows. (B) Arrow indicates rod end-on to the field. Bar = 50 nm.

released by these treatments. The protein electroblotting experiments showed that the major polypeptide and the 70K subunit were cross-reactive with exotoxin A. This finding implies a structural role for these proteins. If the cross-reactive rod proteins are structural components, release of those peptides should be possible only by complete disruption. The extreme stability of the rods, and the retention of the exotoxin A cross-reactive proteins in the rods after a variety of vigorous treatments, suggests that these proteins form an integral part of the rod structure.

DISCUSSION

We have identified and purified particulate structures (rods) by anti-exotoxin A antibody affinity chromatography. The rods were not an artifact of a particular purification protocol. The rods were a consistent component of the supernatant fraction obtained from cultures of *P. aeruginosa* PA103 and could be visualized by electron microscopy. Culture supernatants from mitomycin c-treated cells contained increased numbers of rods, as well as pyocins and bacteriophages. Rods were also present in samples of exotoxin A prepared in different laboratories by a variety of techniques.

Heterologous monoclonal antibody reacted with both antigens, indicating that rods and exotoxin A share common antigenic determinants. The possibility always exists that monoclonal "antirod" antibody was raised against contaminating exotoxin A in the immunogen. However, our data cannot be explained by the trivial contamination of rods by exotoxin A. Monoclonal antitoxin and antirod antibodies bound equivalent amounts of the homologous and heterologous antigen when quantitated in the ELISA. Either antigen was an effective competitor for both monoclonal antibodies. Further, the protein electroblotting experiments demonstrated that monoclonal antibodies to exotoxin A reacted with two of the three rod polypeptides. The cross-reactive polypeptides could not be released from the rod structure unless the rods were totally disrupted by heat and strong detergent. The data suggested that the reactive peptides were an integral part of rods, possibly structural subunits, and were not the result of trivial adsorption of intact exotoxin A to an unrelated structure.

The presence of shared antigenic determinants between exotoxin A and rods may suggest that both forms contain areas of homologous amino acid sequence. Sequence homologies could arise by divergent evolution from a common ancestral gene or through a common biosynthetic pathway. The letter idea would imply the existence of a precursor-product relationship between toxin and rods or, alternatively, that both forms were derived from some common precursor. Processing from a large precursor to a smaller product is often a step in secretion of protein from gram-negative organisms. Our results do not address this point, but analogous pathways have been documented. Cells of *P. aeruginosa*, for a brief period in their growth cycle, contain large amounts of enzymatically inactive, high-molecular-weight material that is related to the major extracellular protease produced by this organism (9). The precursor material is cell-bound and is converted to active protease by limited proteolysis, a process that is apparently a function of the secretory mechanism. Membrane-bound, high-molecular-weight precursors of exotoxin A (P. B. Fernandes and K. R. Cundy, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, B92, p. 32) and a proelastase precursor (B. Wretlind, A. Björklind, I. Karlsson, and A. Hagelberg, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, B86, p. 37) have been reported. Posttranscriptional modification by signal peptidase activity is a general mechanism in the transport of proteins to the periplasmic space or outer membrane of gram-negative bacteria. Secretion of proteins into the extracellular space requires the presence of additional translocational mechanisms.

The uniform morphology and three-subunit composition of rod structure suggest that an assembly process is involved in rod production. The assembly of complex structures such as bacteriophages, flagella, bacteriocins, etc., occurs by two general mechanisms. One pathway requires activation of each subunit by incorporation into a specific intermediate, a process termed self-regulated assembly by King (10). Spontaneous self-assembly is generally promoted by external physical factors such as oxygen, ionic strength, divalent cation concentration, or pH alteration, and often can be reversed by changing the parameters that initiate assembly. We were unable to find conditions other than heat denaturation that cause dissociation of rods into subunits. The observed stability of the rod structure may imply that the assembly of rods is self-regulated, perhaps proceeding by proteolytic processing of the exotoxin A-sized intermediate.

Although rods did not share antigenic determinants with the R-type pyocin of PA103 and were stable to conditions

known to dissociate R pyocin structures, rods had a striking morphological similarity to pyocin or bacteriophage sheaths. Rods may represent the product of a defective pyocin or bacteriophage gene incorporated into an exotoxin A-related operon. The inclusion of a pyocin or bacteriophage promoter into this genetic unit could explain the high concentration of rods in cultures treated with mitomycin c. We do not know how many genes are required for the synthesis of rods. Multiple genetic loci are required for the synthesis and export of exotoxin A (4, 5), although the biosynthetic pathway is not yet fully described. Further studies are necessary to determine whether genes known to function in exotoxin A synthesis are related in any way to the assembly of rods.

We were unable to find any conditions that convert rods to toxin or toxin-like enzymatic activity. Rods are not lethal to mice, or toxic in cultured mouse cells, and this observation demonstrates that rods are either not internalized or are not processed to exotoxin A after internalization. Data to be presented elsewhere indicate that rods are produced in the course of *P. aeruginosa* infection in humans and in mice, and that antibody to rods confers protection from *P. aeruginosa* infection in mice. Rods can be used as a stable nontoxic immunogen to produce antibodies to exotoxin A. Rods are a naturally occurring structure and a major extracellular particulate product of *P. aeruginosa* PA103.

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