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Toxoids of *Pseudomonas aeruginosa* Exotoxin-A: Photoaffinity Inactivation of Purified Toxin and Purified Toxin Derivatives

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For the preparation of greatly detoxified but highly immunogenic toxoids, two enzymatically active, low-toxicity derivatives of *Pseudomonas aeruginosa* exotoxin-A were further inactivated by photoaffinity labeling. These derivatives were formed during toxin purification, when a relatively crude toxin preparation was concentrated by ammonium sulfate precipitation and subsequently dialyzed. These derivatives, designated peak-1 protein (PK-1) and peak-2 protein (PK-2) were antigenically indistinguishable from native toxin, but had isoelectric points (5.00 and 4.90, respectively) that were different from that of the native toxin (4.95). Although the enzymatic activities and molecular weights of PK-1 and PK-2 were similar to those of native toxin, their toxicities were greatly reduced (ca. 500-fold). Photoaffinity labeling of fully active toxin-A, purified by a process which limits the formation of these derivatives, decreased its enzymatic activity (ca. 30-fold) and toxicity (ca. 100-fold). Likewise, photoaffinity labeling of purified PK-1 and PK-2 decreased their enzymatic activities and toxicities (ca. 30-fold and 100-fold, respectively) and, thus, yielded toxoids that were ca. 50,000-fold less toxic than unpurified native toxin. These toxoids were irreversibly detoxified and highly immunogenic during 9 months of storage at 4°C.

Recent evidence suggests that exotoxin-A is an important virulence factor in the pathogenesis of *Pseudomonas aeruginosa* infections (5, 11, 22, 25, 26). Toxin-A is a proenzyme that is thought to be converted to active enzyme within susceptible mammalian cells. Like diphtheria toxin, it catalyzes the transfer of the ADP-ribose moiety of NAD onto elongation factor 2, a translocating enzyme required for eucaryotic polypeptide chain elongation (12). This covalent attachment leads to inhibition of protein synthesis and cell death. Besides the enzymatically active region ("A" region), toxin-A is believed to contain a binding region ("B" region) that is necessary for toxin entry into cells (17, 29). Thus, in theory, functional alterations of either of these regions would decrease the toxicity of the molecule.

Attempts by other investigators to convert toxin-A to a toxoid by chemical means have relied primarily on the use of traditional nonspecific inactivating reagents, such as glutaraldehyde and formaldehyde, which generally produce toxoids of toxin-A that have poor immunogenicity or that tend to revert to the toxic state (6, 7, 23, 25; S. H. Leppla, O. C. Martin, and O. R. Pavlovskis; Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, B96, p. 29). We took a new approach to the detoxification of toxin-A, that is, the inactivation of its enzymatic (A region) activity by the specific process of photoaffinity labeling. In this process, a photolabile azido substrate analog is converted by light to a short-lived, reactive, nitrene intermediate that forms covalent bonds with the substrate-binding portion of the enzyme, while leaving the remainder of the molecule unaltered. It was postulated that, although intact toxin-A requires denaturation and reduction for expression of full enzymatic activity in vitro (12, 29), a substrate-binding site might exist on the unmodified molecule which would permit affinity labeling. Indeed, we found that photoaffinity labeling of toxin-A with analogs of NAD does reduce its enzymatic activity (19).

In this report, we present evidence that photoaffinity labeling of purified native toxin reduces its enzymatic activity and toxicity in a parallel manner. We also describe a purification process that converts toxin-A into two low-toxicity derivatives, designated peak-1 protein (PK-1) and peak-2 protein (PK-2). These derivatives had isoelectric points which were different from each other and from that of native toxin. However, PK-1 and PK-2 were antigenically indistinguishable from native toxin, and they had enzymatic activities and molecular weights similar to that of native toxin. Thus, the formation of these low-toxicity derivatives may result from an alteration(s) in the B region. Photoaffinity labeling of PK-1 and PK-2 yielded greatly detoxified toxins that were stable and highly immunogenic.

MATERIALS AND METHODS

Stock cultures of *P. aeruginosa* PA103 (strain 29260, American Type Culture Collection, Rockville, Md.) were prepared as described previously (1) and stored at −70°C.

Purified native toxin. For the preparation of purified native toxin-A, we used essentially the culture conditions and purification process described by Leppla (15). As recommended by Leppla (personal communication), the purification process was modified by the use of β-mercaptoethanol throughout most of the process (D. R. Galloway, R. C. Hedstrom, and O. R. Pavlovskis, manuscript in preparation).

Growth medium. Enriched growth medium, used for the preparation of the crude toxin described below, was prepared as follows: iron was removed from tryptic soy broth by mixing 1.8 kg of dry medium with 600 g of Chelex-100 (Bio-Rad Laboratories, Richmond, Calif.) in 5.4 liters of deionized water for 6 h at room temperature (13). The broth

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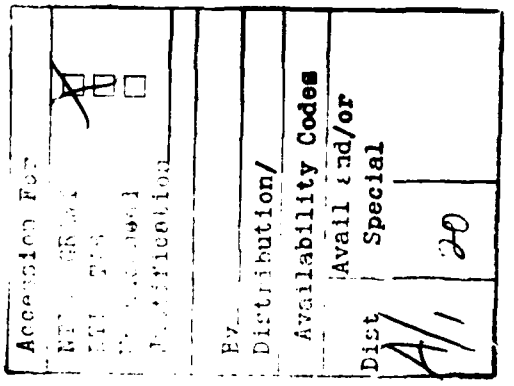

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then was filtered through Whatman no. 1 filter paper (Whatman Chemical Separation, Inc., Clifton, N.J.), diluted to 60 liters with deionized water, and diafiltered through an H10P10 hollow fiber cartridge (DC-30 system; Amicon Corp., Danvers, Mass.) to yield 40 liters of deferrated growth medium. The medium was sterilized by filtration (0.45-μm pore size) and enriched just before use with sterile solutions of 1 M monosodium glutamate and 20% glycerol to give final concentrations of 0.05 M and 1%, respectively (16).

Crude toxin. Crude toxin, which contained native toxin-A, was prepared as follows: four tryptic soy agar (Difco Laboratories, Detroit, Mich.) slants were streaked with stock culture. After incubation at 37°C for 18 h, the growth from the slants was used to inoculate 400 ml of enriched growth medium. The culture was shaken (250 oscillations per min) for 6 h at 32°C and was used to inoculate 40 liters of enriched medium. Fermentation proceeded overnight in a Bilthoven-SuperPaljas-NW200 40-liter unit (Contact-Roestrijstaal, Rotterdam, The Netherlands). The culture was stirred at 32°C and maintained at a dissolved-oxygen level of not less than 25% saturation. The overnight culture was clarified by centrifugation, and the supernatant fluid was sterilized by filtration (0.45 μm). The volume of the supernatant fluid (kept at 4°C) was reduced 10-fold by diafiltration with an Amicon DC-10 hollow fiber system and an H10P10 cartridge. The concentrated retentate, designated crude toxin, was used directly for the preparation of PK-1 and PK-2 or was stored at −70°C for toxicity studies.

PK-1 and PK-2. PK-1 and PK-2 were obtained by the following purification process (counterimmunoelectrophoresis was used to monitor toxin antigen): a 4-liter sample of crude toxin was diluted with 10 liters of cold deionized water and subjected to batch absorption at 4°C overnight with 2 liters of diethylaminoethyl cellulose (Whatman DE-52) as described by Leppla (15). Toxin antigen that eluted with the 0.25 M NaCl wash (2.5 liters) was precipitated from solution by slowly adding solid $(NH_4)_2SO_4$ to 70% saturation, with constant stirring. The precipitate was allowed to form overnight at 4°C. It then was divided into three equal portions, and each, in sequential order, was collected by centrifugation, dissolved in 30 ml of column buffer (0.5 M NaCl, 0.1 M Tris-hydrochloride, 0.02% NaN_3; pH 8.0) and chromatographed at 4°C through a column (5 by 100 cm) packed with Sephadex G-75 (Pharmacia Fine Chemicals, Piscataway, N.J.). Fractions were assayed for toxin antigen, pooled, and concentrated by precipitation with $(NH_4)_2SO_4$ as before. The next step in purification, hydroxylapatite chromatography, was performed as described previously (1) with a packed column (2.6 by 40 cm) maintained at 4°C. Hydroxylapatite-purified toxin antigen was concentrated by precipitation via dialysis against saturated ammonium sulfate at 4°C. The resultant precipitate was removed from the dialysis membrane, collected by centrifugation, and dissolved in 5 ml of gradient-starting buffer (0.05 M NaCl, 0.01 M Tris, pH 8.0). The toxin antigen was dialyzed exhaustively against this buffer at 4°C. It then was applied to a column (1.6 by 40 cm) of Whatman DE-52 and chromatographed at room temperature with a linear gradient of NaCl (0.05 to 0.5 M) in Tris buffer. The toxin-antigen-containing fractions corresponding to PK-1 and PK-2 were pooled separately, filter sterilized (0.45 μm), and stored at −70°C.

Toxoid production. Purified native toxin-A, PK-1, and PK-2 were converted to toxoids by the photoaffinity inactivation procedure described previously (19). Briefly, toxin solution (150 μg/ml of phosphate-buffered saline [PBS]) was mixed with an equal volume of substrate analog solution (2.6 mM 8-azidoadenosine in PBS), placed under N_2 in a Pyrex reactor kept at 0°C, and then irradiated for 6 min with a high-pressure quartz mercury-vapor lamp. Unbound 8-azidoadenosine was removed from the resultant toxoid solution by dialysis against saline.

Antisera. Anti-native toxin was prepared by subcutaneous immunization of rabbits (2). Anti-PK-1-PK-2 was prepared by the intramuscular hyperimmunization of horses with a mixture (800 μg total) of PK-1 and PK-2 (not affinity labeled) admixed with Freund incomplete adjuvant. Anti-PK-1 toxoid was produced by the subcutaneous hyperimmunization of rabbits with PK-1 toxoid (150 μg total) admixed with Freund incomplete adjuvant.

Qualitative immunoassays for toxin antigen. Counterimmunoelectrophoresis was performed with an antigen II counterimmunoelectrophoresis system (Hyland Diagnostics, Costa Mesa, Calif.) and horse anti-PK-1-PK-2. Pattern D agar plates (Hyland Diagnostics, Division of Travenol Laboratories, Inc., Deerfield, Ill.) were used for double immunodiffusion.

Quantitative immunoassay for toxin antigen. Quantitative (rocket) immunoelectrophoresis of toxin antigen was performed by using either 1% rabbit anti-native toxin or 0.4% horse anti-PK-1-PK-2 in agarose according to the procedure described elsewhere (32). Purified native toxin-A samples of known protein concentration were used to construct standard curves.

Protein measurements. Protein concentrations were determined by the method of Lowry et al. (18), with serum albumin (Pentex; Miles Laboratories, Inc., Elkhart, Ind.) as the standard.

Enzyme assay. ADP-ribosyl transferase activity of duplicate samples was measured according to the method described by Iglewski and Sadoff (13), except that 0.5 μM adenine-[^{14}C]NAD was used instead of 5.0 μM. The precipitable products of the reaction were counted by liquid scintillation, and the specific activity (counts per minute per microgram of toxin antigen) was calculated.

In vitro cytotoxicity assay. Inhibition of thymidine incorporation in mouse L-929 fibroblasts (L-cells) (14) was used to measure the cytotoxic activity of toxins and toxoids. Test samples, diluted in medium, were filter sterilized with a 0.45-μm polycarbonate membrane (Bio-Rad), and 0.1-ml portions (twofold dilutions) were added to five replicate wells. The amount of [^{3}H]thymidine incorporated was determined by liquid scintillation, and the 50% inhibition dose was calculated (24).

Mouse lethality assay. Mouse toxicity tests were performed as described by Iglewski and Sadoff (13). Groups of five 4-week-old female Swiss Webster mice (CAMM, Wayne, N.J.) were given graded doses (twofold dilutions in 0.2 ml of PBS) of test material intravenously, and the medium lethal dose (LD_{50}) was determined.

Antigen-extinction immunogenicity assay. Three-week-old mice were injected intraperitoneally with graded doses of alum-adsorbed protein (PK-1 or PK-1 toxoid) on days 0 and 21. On day 35, some of the mice from each group were bled for antibody assay, and the remainder were challenged intravenously with 288 ng (four LD_{50}s for 8-week-old mice) of crude toxin in 0.2 ml of PBS.

Enzyme-linked immunosorbent assay. The serum antibody responses of mice to PK-1 antigen was measured in duplicate by enzyme-linked immunosorbent assay (30) with an automated EIA-PR50 instrument (Gilford Laboratories, Inc., Oberlin, Ohio). A positive antibody response was indicated

by an optical density that was greater than two times that of the background at a 1:40 dilution of serum.

Conventional PAGE and SDS-PAGE. Conventional polyacrylamide gel electrophoresis (PAGE) was performed by the method of Davis (8) with a 4% stacking gel and a 7% separating gel. Sodium dodecyl sulfate (SDS)-PAGE was performed by the method of Weber and Osborn (31) with a 3% stacking gel and a 10% separating gel. For SDS-PAGE, protein samples were solubilized by being boiled in a solution containing 2% SDS and 5% β-mercaptoethanol and then were electrophoresed in the presence of 0.1% SDS. An SE-600 series vertical slab gel unit (Hoefer Scientific Instruments, San Francisco, Calif.) was used for both PAGE and SDS PAGE. Silver stain (21) was used for all gels.

Isoelectric focusing. Protein samples (250 μg/ml) were dialyzed against 1% glycine and then 2 μl of the dialysand was subjected to isoelectric focusing in an Ampholine PAGplate (pH 4.0 to 6.5) mounted on a flatbed 2217 Ultrophor electrofocusing unit (LKB Instruments, Inc., Gaithersburg, Md.). The samples were focused for 2.5 h at 10°C and at a constant power setting of 25 W. The pH gradient was determined with a surface electrode, and then the gel was silver stained by the method by Morrissey (21).

RESULTS

Effects of the purification process on enzymatic activity and toxicity of toxin-A. In the final step of our purification process, anion-exchange chromatography, two distinct peaks of toxin antigen were obtained. These products were designated PK-1 and PK-2 according to their sequential elution from the column (Fig. 1). When compared with crude toxin (Table 1), both PK-1 and PK-2 were greatly reduced in toxicity for mice (at least 100-fold) and L-cells (at least 500-fold), whereas purified native toxin (obtained by a different process [15]) had a relatively small loss of toxicity (about 2-fold) for mice and L-cells. The loss of toxicity in PK-1 and PK-2 was obtained in 12 separate purification runs. However, as in the case of purified native toxin, the enzymatic activities of these proteins were reduced only minimally (two- to threefold) (Table 1). Like crude toxin, PK-1 and PK-2 had to be activated with a reducing agent and a denaturant for the expression of full enzymatic activity. At least a 10-fold increase in enzymatic activity (counts per minute per microgram of antigen) occurred due to activation.

Isoelectric points of PK-1, PK-2, and native toxin-A. PK-1 preparations had a major band with a pI of 5.00; native toxin preparations had a major band with a pI of 4.95; and PK-2 preparations had a major band with a pI of 4.90 (Fig. 2). These differences in electrical charge account for the sequential elution of PK-1 and PK-2 from the DEAE anion-exchange column (Fig. 1) and for the different electrophoretic mobilities obtained in conventional PAGE with all three of these proteins (see below).

Formation of PK-1 and PK-2 during isolation. We examined samples from various points along the purification

TABLE 1. Enzymatic activity and toxicity of native toxin-A, PK-1, and PK-2

Preparation[a]	Enzymatic activity[b] (10^3 cpm/μg of antigen)	Toxicity	
		L-cell ID_{50}[c] (pg of antigen/10^3 cells)	Mouse LD_{50} (ng of antigen/mouse)
Crude toxin	94.0 ± 5.8	7	30.5
Purified proteins			
Native toxin	54.5 ± 4.8	11	56.1
PK-1[d]	32.5 ± 2.8	3,760	41,700
PK-2[d]	45.7 ± 4.0	3,550	35,600

[a] Antigen concentrations were determined by rocket immunoelectrophoresis with purified native toxin as the antigen standard.

[b] ADP-ribosyl transferase activity (mean ± standard error of the mean of two determinations) was determined for samples in PBS that contained a reducing agent and a denaturant (activated samples).

[c] ID_{50}, 50% inhibition dose.

[d] PK-1 and PK-2 were separated by DE-52 ion-exchange chromatography.

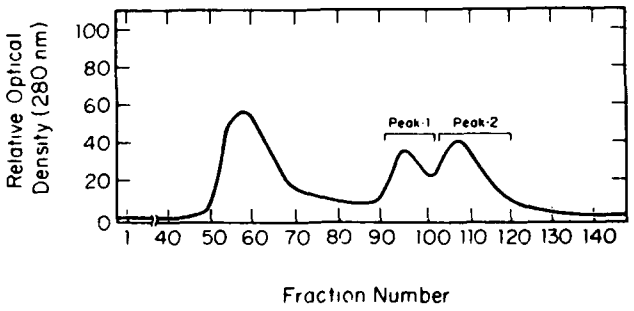

FIG. 1. Purification of PK-1 and PK-2 by Whatman DE-52 anion-exchange chromatography. Column fractions (2.5 ml) were monitored for protein by spectrophotometry (——) and for toxin antigen by counterimmunoelectrophoresis. Toxin antigen was found only in optical density peaks 1 and 2, with concentrations that reflected the relative optical densities within these peaks. Fractions corresponding to peaks 1 and 2 were pooled as indicated by the brackets.

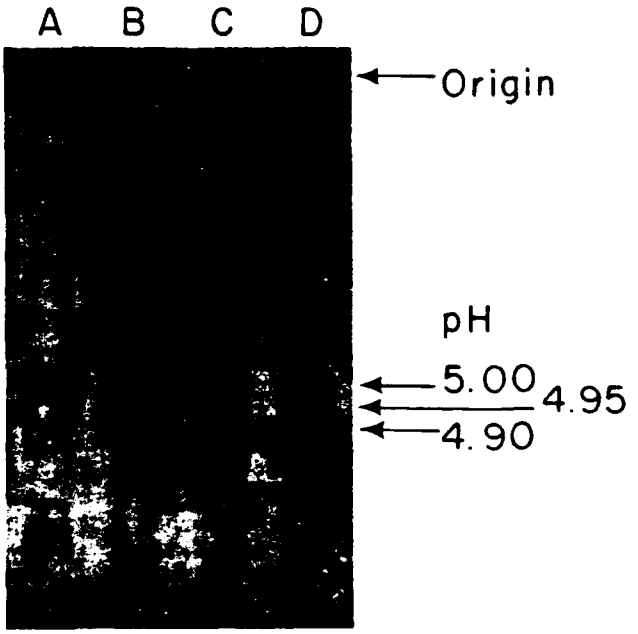

FIG. 2. Isoelectric focusing of purified PK-1 (lane A), native toxin-A (lane B), PK-2 (lane C), and a mixture of all three proteins (lane D). After focusing, the pH gradient (4.0 to 6.5) was determined by a surface electrode. The isoelectric points are indicated by arrows.

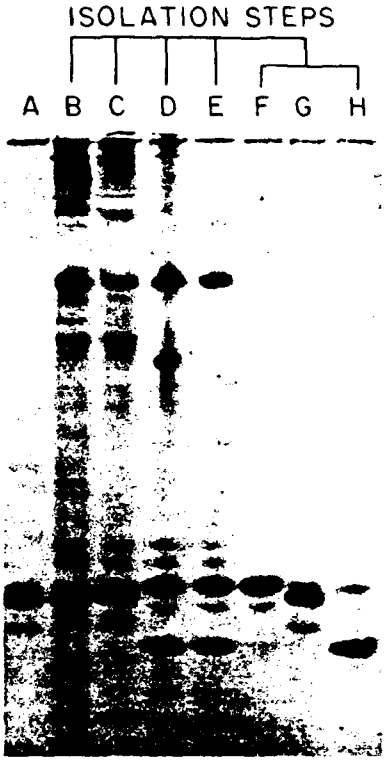

FIG. 3. Conventional PAGE illustrating the conversion of native toxin-A to its derivatives (PK-1 and PK-2) during the purification process. The following samples (1.0 μg to 1.5 μg of toxin antigen each) were electrophoresed anodally (from top to bottom): purified native toxin (lanes A and G) and samples from progressive stages in the purification process, that is, crude toxin (lane B), Whatman DE-52 batch absorption product (lane C), Sephadex G-75 gel filtration product concentrated by ammonium sulfate precipitation (lane D), hydroxylapatite-chromatography product (lane E), and the final Whatman DE-52 anion-exchange chromatography products designated PK-1 (lane F) and PK-2 (lane H). Note the absence of native toxin and the presence of PK-1 and PK-2 in lanes D and E. To increase the separation of the proteins, electrophoresis was allowed to continue for a total of 7.67 h. This long running time caused two major fast-migrating proteins, which normally appear in the patterns of crude toxin and the Whatman DE-52 batch absorption product, to migrate out of the gel.

process for the presence of PK-1 and PK-2 by conventional PAGE. With the samples available from one purification run (Fig. 3), it was possible to determine that native toxin was virtually replaced by PK-1 and PK-2 by the end of the fifth isolation step (ammonium sulfate precipitation and dialysis of the Sephadex G-75 filtration product). In a subsequent purification run (data not shown), native toxin was present in the Sephadex G-75 product before ammonium sulfate precipitation but was virtually replaced by PK-1 and PK-2 after ammonium sulfate precipitation and dialysis. This indicates that native toxin was altered to form these proteins at this concentration step. In the purification runs examined, there remained sufficient quantities of native toxin in PK-1 and PK-2 preparations to be detectable by isoelectric focusing (Fig. 2) and conventional PAGE (Fig. 3). The loss of native toxin was accompanied by ca. 500-fold reduction in L-cell toxicity.

Our data also indicate that some PK-1 already existed in the culture supernatants. In conventional PAGE (Fig. 3), crude toxin preparations contained a minor protein band corresponding to the major band in PK-1 preparations. This band was present in all subsequent isolation steps (Fig. 3) and, as one might expect from the Whatman DE-52 profile (Fig. 1), it appeared as a minor band in PK-2 preparations (Fig. 2 and 3). Similarly, native toxin preparations, purified by a different process, contained small amounts of PK-1 (Fig. 2 and 3).

Effect of photoaffinity labeling on enzymatic activity and toxicity of native toxin-A, PK-1, and PK-2. Photoaffinity labeling of purified native toxin, PK-1, and PK-2 decreased their enzymatic activity by ca. 30-fold and their toxicity for L-cells by ca. 100-fold (Table 2). The mouse lethality of native toxin was decreased 37-fold. The toxoids remained inactivated when stored at 25°C (Table 2) or 4°C (data not shown) for 2 weeks. A PK-1 toxoid preparation, which was stored at 4°C, remained inactivated for 10 months (data not shown).

As a control for the photoaffinity process, samples of each protein solution were mixed with the photolabile reagent but were not irradiated with light. As in the case of the irradiated samples, the unbound substrate analog was removed by dialysis. These "toxoid controls" had enzymatic activities and toxicities similar to those of the respective unprocessed proteins (Table 2). Other control materials (not shown) which were exposed to irradiation but not to photolabile reagent also had undiminished activities (19).

Physicochemical properties of native toxin-A, PK-1, PK-2, and the photoaffinity-derived toxoids. In SDS-PAGE, which separates proteins on the basis of size alone, the patterns obtained with native toxin-A, PK-1, and PK-2 and their respective photoaffinity-derived toxoids were essentially the same (Fig. 4). All of these proteins had a molecular mass of ca. 67,000 daltons.

In conventional PAGE, which separates proteins on the basis of charge and size, the photoaffinity-derived toxoids tended to have slightly greater electrophoretic mobilities (greater negative charge) and diffuse banding (greater charge heterogeneity) as compared with their respective controls (Fig. 5).

Antigenic properties of native toxin-A, PK-1, PK-2, and the photoaffinity-derived toxoids. When compared by double immunodiffusion, PK-1, PK-2, and native toxin formed a line of identity with antiserum prepared against purified native toxin (Fig. 6A) and with antiserum against a combination of PK-1 and PK-2 (data not shown). These proteins also were indistinguishable by quantitative immunoelectrophoresis (Table 3).

Photoaffinity-labeled toxoids of native toxin, PK-1, and PK-2 formed a line of identity with native toxin-A when tested with antiserum prepared against native toxin (data not shown) and antiserum prepared against PK-1 toxoid (Fig. 6B). These toxoids also were indistinguishable from crude toxin by this method (data not shown). By quantitative immunoelectrophoresis, a minimal loss (ca. 13%) of antigenicity was observed in the affinity-labeled proteins as compared with their unprocessed counterparts (Table 3). However, similar losses of antigenicity (ca. 11%, on the average) were observed in the toxoid controls. This suggests that most, if not all, of the loss of antigenicity in the toxoids was due to the handling of the proteins and not to the covalent bonding of the substrate analog.

Immunogenicity of photoaffinity-derived toxoid. Because a greater degree of detoxification was obtained when our

FIG. 5. Conventional PAGE of purified native toxin-A, PK-1, PK-2, and their respective photoaffinity-derived toxoids. The following preparations (1.0 to 1.5 μg of protein) were electrophoresed anodally (from top to bottom): unprocessed PK-1 (lane A), toxoid control of PK-1 (lane B), toxoid of PK-1 (lane C), toxoid control of native toxin (lane D), toxoid of native toxin (lane E), toxoid control of PK-2 (lane F), and toxoid of PK-2 (lane G). Note that the toxoid bands smeared anodally (lanes C, E, and G).

tation. This derivative also has an isoelectric point different from that of native toxin but, unlike PK-1 and PK-2, it has only partial antigenic identity with native toxin. The disparity observed between the small loss of enzymatic activity and the great loss of toxicity in PK-1 and PK-2 suggests that the receptor-binding portion (B region) of native toxin is altered during the formation of these derivatives. Such an alteration(s) might affect receptor-mediated binding or subsequent processing of toxin, both of which are important for toxicity (9, 10, 27).

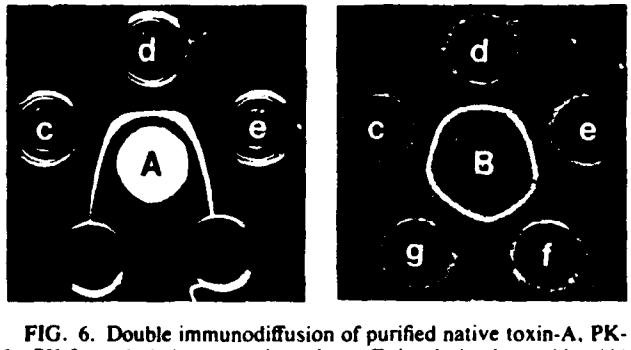

FIG. 6. Double immunodiffusion of purified native toxin-A, PK-1, PK-2, and their respective photoaffinity-derived toxoids. (A) Rabbit anti-native toxin was run against samples (0.88 to 1.00 μg of protein per well) of PK-1 (well c), native toxin (well d), and PK-2 (well e). (B) Rabbit anti-PK-1 toxoid was run against samples (0.22 to 0.25 μg of protein per well) of native toxin (wells e and g) and photoaffinity-derived toxoids of PK-1 (well c), native toxin (well d), and PK-2 (well f).

Conversion of native toxin-A to PK-1 and PK-2 occurred when a partially purified preparation of toxin (the Sephadex G-75 product) was concentrated by ammonium sulfate precipitation and then dialyzed. Purified native toxin could not be converted to these derivatives in this manner (unpublished data), which suggests that the conversion of native toxin is mediated by factors, possibly enzymes, that are present in the relatively crude Sephadex G-75 product and are highly concentrated by ammonium sulfate precipitation. In another experiment, the formation of PK-2 (but not PK-1) was prevented by the addition of β-mercaptoethanol to the solutions (unpublished data). Although this may suggest that PK-2 is formed by oxidation of PK-1 or native toxin, we do not have sufficient data to determine the chemical basis for the formation of either PK-2 or PK-1.

Although our purification process does not differ greatly from previously described procedures (1, 2, 13, 15), its use consistently resulted in the conversion of native toxin to PK-1 and PK-2. This may be due to the fact that our process does not employ high-resolution isolation steps, such as gradient elution ion-exchange chromatography, early in the procedure. Such high-resolution steps may separate the converting factors (e.g., enzymes) from native toxin. In the purification runs that we chose to analyze, PK-1 and PK-2 were not formed at the first concentration step (ammonium sulfate precipitation of the Whatman DE-52 batch absorption product). This may be due to the relatively short time that the toxin is exposed to the highly concentrated converting factors at this step, or it may be that the toxin in the Whatman DE-52 product is protected from these factors by other impurities which are removed later by Sephadex G-75 gel filtration.

The photoaffinity-derived toxoids exhibited several properties that might be expected as the result of specific covalent bonding of substrate analog to the enzymatically active site. Their enzymatic activities and toxicities were irreversibly inactivated, but their physicochemical properties and antigenic properties were similar to those of native

TABLE 3. Antigenicity of photoaffinity-derived toxoids of purified native toxin-A, PK-1, and PK-2

Preparation[a]	Protein concn[b] (μg/ml)		Relative antigenicity[c] (rocket/chemical)
	Chemical assay	Rocket assay[d]	
Native toxin			
Unprocessed	215	Std	1.00
Toxoid control	110	105	0.95
Toxoid	108	93	0.86
PK-1			
Unprocessed	234	233	1.00
Toxoid control	113	96	0.85
Toxoid	114	99	0.87
PK-2			
Unprocessed	252	279	1.11
Toxoid control	116	115	0.99
Toxoid	123	120	0.98

[a] For toxoid controls, the protein solutions were mixed with the photolabile reagent but were not irradiated with light.

[b] The protein concentration of fresh preparations was determined by chemical (Lowry) assay and by rocket immunoelectrophoresis.

[c] Protein concentration determined by rocket assay was divided by the concentration determined by chemical assay.

[d] Horse anti-PK-1-PK-2 was used for the rocket assay. Unprocessed purified native toxin was used as the antigen standard (Std).

TABLE 2. Effects of photoaffinity labeling on enzymatic activity and toxicity of purified native toxin-A, PK-1, and PK-2[a]

Preparation[b]	Enzymatic activity (10^3 cpm/μg of antigen)	Toxicity	
		L-cell ID_{50}[c] (pg of antigen/10^3 cells)	Mouse LD_{50} (ng of antigen/mouse)
Native toxin			
Unprocessed	54.5 ± 4.8	11	56
Toxoid control	30.4 ± 8.3	11	67
Toxoid control (25°C)	31.1 ± 6.2	10	ND[d]
Toxoid	1.6 ± 0.2	1,440	2,080
Toxoid (25°C)	1.6 ± 0.1	1,330	ND
PK-1			
Unprocessed	32.5 ± 2.8	3,760	41,700
Toxoid control	18.4 ± 10.6	2,030	ND
Toxoid control (25°C)	39.5 ± 5.9	3,380	ND
Toxoid	1.2 ± 0.3	571,000	ND
Toxoid (25°C)	1.7 ± 0.2	595,000	ND
PK-2			
Unprocessed	45.7 ± 4.0	3,550	35,600
Toxoid control	19.4 ± 4.2	3,440	ND
Toxoid control (25°C)	31.2 ± 3.5	3,240	ND
Toxoid	1.6 ± 0.1	299,000	ND
Toxoid (25°C)	3.7 ± 0.5	219,000	ND

[a] ADP-ribosyl transferase activity and toxicity were determined as described in Table 1, footnote *b*.
[b] Affinity-labeled toxoids and toxoid controls (described in the text) were tested for enzymatic activity and toxicity when freshly prepared and after storage for 1 week at 25°C.
[c] ID_{50}, 50% inhibition dose.
[d] ND, Not done.

isolation process was combined with the photoaffinity process, we chose to investigate in detail the immunogenicity of affinity-labeled PK-1. The choice of PK-1 over PK-2 was arbitrary. Rabbit antiserum against PK-1 toxoid neutralized the enzymatic activity (Fig. 7A), L-cell cytotoxicity (Fig. 7B), and mouse lethality (Fig. 7C) of crude toxin, whereas preimmunization serum had no effect.

The capacity of labeled and unlabeled PK-1 to elicit antibody production and to protect mice against a lethal challenge with crude toxin was evaluated. The comparison was made possible by the greatly reduced toxicity of PK-1 before photoaffinity inactivation. The median effective dose for both preparations, as determined by serum antibody and protection against toxin, was two injections of ca. 1 μg of alum-adsorbed antigen (Table 4). This indicates that the two proteins were similar in immunogenicity. The two measures correlated well (Spearman's rho = 0.93; $P = 0.0026$).

The immunogenicity of the affinity-labeled toxoid appeared to be stable at 4°C. For example, when the immunogenicity of one lot of alum-adsorbed PK-1 toxoid was measured by antigen extinction for 9 months, the capacity to induce antibodies and protection against crude toxin did not decline during this time (Table 5).

DISCUSSION

The data presented here indicate that PK-1 and PK-2 are enzymatically active but poorly toxic derivatives of native toxin-A. Unlike the nontoxic 26,000-dalton and 48,000-dalton fragments described by other investigators (3, 17, 20, 28, 29), these derivatives were similar in molecular mass to the native toxin (67,000 daltons) and were antigenically indistinguishable from it. Coleman and Arbuthnott (4) have reported a nontoxic cross-reactive derivative of toxin-A that, like PK-1 and PK-2, is formed when crude toxin preparations are concentrated by ammonium sulfate precipi-

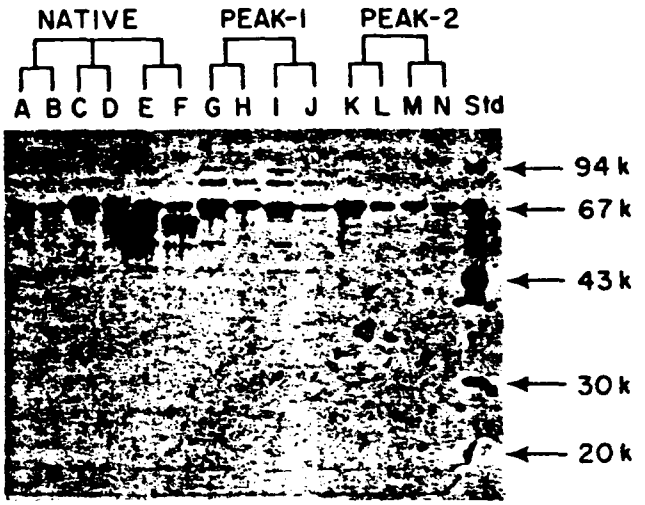

FIG. 4. SDS-PAGE of purified native toxin-A, PK-1, PK-2, and their respective photoaffinity-derived toxoids. Each of the following samples was run at two levels (3.0 and 1.5 μg of protein): unprocessed native toxin (lanes A and B), toxoid of native toxin (lanes C and D), toxoid control of native toxin (lanes E and F), toxoid of PK-1 (lanes G and H), toxoid control of PK-1 (lanes I and J), toxoid of PK-2 (lanes K and L), and toxoid control of PK-2 (lanes M and N). The molecular weights of the standards (Std) are given in kilodaltons (k). For toxoid controls, the protein solutions were mixed with the photolabile reagent but were not irradiated with light.

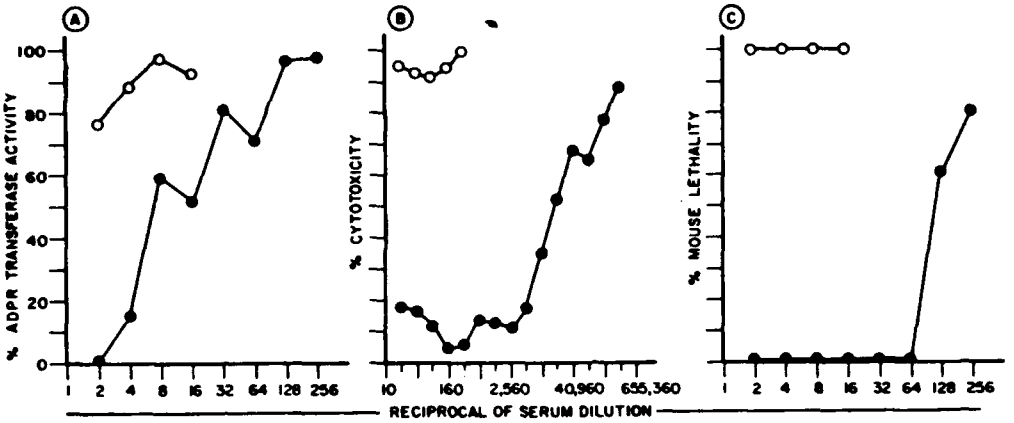

FIG. 7. Effect of rabbit anti-PK-1 toxoid serum on (A) ADP-ribosyl transferase activity, (B) L-cell cytotoxicity, and (C) mouse lethality of crude toxin. Preimmunization (○) and post-immunization (●) sera were serially diluted in PBS, mixed with equal volumes of crude toxin solutions (total dilutions of sera are shown), and then incubated at 37°C for 30 min. The mixtures contained 60 ng of toxin antigen in 10 μl for the enzyme assay, 33 pg in 0.1 ml for the cytotoxicity assay, and 144 ng in 0.2 ml for the 4-week-old mouse assay.

toxin. Our results indicate that the efficiency of the photoaffinity inactivation process is not affected by the use of PK-1 and PK-2, e.g., the enzymatic activities and toxicities of these proteins were decreased to the same extent (ca. 30- and 100-fold, respectively) as that of the purified native toxin.

From the available data, we cannot determine the source of the residual toxicity present in PK-1, PK-2, or their respective toxoids. It is possible that PK-1 and PK-2 have inherent residual toxicity. It is equally possible that the derivatives are completely inactivated and that the residual toxicity is due to the presence of a trace amount of native toxin that copurifies with them (Fig. 2, 3, and 5). The residual enzymatic activity (19) and toxicity observed after photoaffinity labeling may likewise be due to a few unaffected molecules or to the retention of some activity by all of the molecules.

TABLE 4. Immunogenicity of PK-1 and its photoaffinity-derived toxoid[a]

Antigen (μg/mouse)	Seroconversion		Protection	
	No. positive/total	ED_{50}[b]	No. of survivors/total	ED_{50}
PK-1				
5	3/4		8/8	
1.5	2/4	1.51 (0.53, 6.34)	5/10	0.95 (0.31, 3.11)
0.5	1/5		4/10	
0.15	0/5		0/10	
PK-1 toxoid				
5	5/5		9/10	
1.5	3/4	0.99 (0.54, 1.87)	7/10	1.04 (0.58, 1.93)
0.5	1/5		2/10	
0.15	0/5		0/10	

[a] Nine groups of mice were injected on days 0 and 21 with the doses of alum-adsorbed antigens shown or with alum alone. On day 35, some mice were tested for production of antibodies by enzyme-linked immunosorbent assay (the serum was positive at a 1:40 dilution), and the others were challenged with four LD_{50}s of crude toxin. Deaths were monitored for 5 days after challenge. Mice that were injected with alum alone did not produce antibodies or survive challenge.

[b] The median effective dose (ED_{50}) (95% confidence limits), when given twice, was estimated by probit analysis.

The antigenicity of the toxoids of PK-1 and PK-2 was not significantly altered despite the great lack of toxicity (ca. 50,000-fold less than crude toxin). The immunogenicity of these toxoids was demonstrated by the ability of anti-toxoid sera to neutralize the enzymatic activity, L-cell cytotoxicity, and mouse lethality of crude toxin. Photoaffinity-labeled and unlabeled PK-1 were equal in immunogenicity, as determined by the ability to induce antibodies and protection against toxin. The immunogenicity of one lot of PK-1 toxoid appeared unchanged over a 9-month period of storage at 4°C, which suggests long-term stability at this temperature. Thus, it appears that photoaffinity labeling of PK-1 or PK-2 may provide an effective and safe toxoid vaccine for use in humans.

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TABLE 5. Antigenic stability of the photoaffinity-derived toxoid of PK-1

Mo. post-affinity labeling[a]	Median effective dose[b] (μg/mouse)	
	Antibody	Protection
0	1.11 (0.52, 2.94)	1.69 (0.89, 3.38)
3	2.76 (1.36, 6.20)	2.62 (1.33, 5.10)
5	1.18 (0.49, 2.61)	1.96 (0.89, 4.66)
9	0.67 (0.08, 2.31)	3.43 (1.76, 6.30)

[a] A preparation of photoaffinity-labeled PK-1 was alum adsorbed, stored at 4°C, and tested for immunogenicity at the indicated times.

[b] Groups of 16 to 20 mice each were injected twice with graded doses of the alum-adsorbed toxoid as described in Table 4, footnote *a*. Half of the mice were tested for production of antibodies; the other half were tested for survival of challenge with crude toxin. The median effective dose (95% confidence limits), when given twice, was estimated by probit analysis.

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