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Purification and Characterization of Different Types of Exfoliative Toxin from *Staphylococcus aureus*

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Exfoliative toxin was isolated from strain DI of *Staphylococcus aureus* using carboxymethyl cellulose and hydroxylapatite chromatography. This purified toxin was compared with that produced by strain TA. The specific biological activity of the two toxins was the same, but they were serologically distinct. These strains have different loci (plasmid or chromosomal) for toxin production; differences were seen in molecular weight and amino acid composition. N-terminal amino acid sequences from the two strains showed significant homology using a single unit alignment shift.

Staphylococcal exfoliative toxin has been implicated in clinical cases of scalded-skin syndrome (3, 4, 6, 11, 17). Symptoms of disease may include erythema, itching, and localized bullae or may be characterized by widespread exfoliation covering a large portion of the body. Ritter von Rittershain (19) in 1878 first described the syndrome in children, and later Lyell (16) reported similar findings in adults. Other workers have described the same findings, and many have isolated *Staphylococcus aureus* from these patients. Generally these organisms belong to phage group II, although there are some toxin producers in other phage groups (14).

Melish and Glasgow first reported an experimental model for studying the biological action of exfoliatin (17). Injection of culture supernatants from toxin-producing staphylococci in newborn mice causes formation of a characteristic Nikolsky sign. Several groups have reported methods for purifying the product responsible for this syndrome (2, 4, 10, 13, 17, 26). Various strains have been employed for toxin production, and the resulting purified preparations have been somewhat different. Distinct types of exfoliatin have been reported by Kondo et al. (13), Arbuthnott et al. (3) and Wiley and Rogolsky (26). When we initially purified exfoliatin from strain TA, its relationship to other types of exfoliatin was uncertain. We have now purified toxin from another strain (DI), selected for study because genetic control of toxin production is reported to be located on a plasmid (21) as compared with strain TA, in which control is on the chromosome. We report here on some similarities between the two types of toxin, as well as some differences, including the primary structure of the amino-terminal segments of the molecules.

MATERIALS AND METHODS

Bacterial strains. *S. aureus* TA and DI were obtained from the laboratory of Rogolsky, Kansas City, Mo. (20). Both strains produce alpha-hemolysin, are coagulase positive, and belong to phage group II.

Medium. Cultures were propagated from lyophilized ampoules in liquid medium as previously described (10). Solid medium employed in these studies was Trypticase soy agar (Baltimore Biological Laboratory, Cockeysville, Md.).

Fermentation. Large-scale production of toxin was achieved by growing the bacteria in 50 liters of Trypticase-yeast liquid medium in a 70-liter fermentor (Fermentation Design, New Brunswick, N.J.). Optimal conditions used were 400 rpm and a constant sparge of 10 liters/min with 90% air and 10% CO_2. Cultures were grown for 18 to 20 h at 37°C and then centrifuged to remove bacteria. Culture supernatants were concentrated at 4°C with an Amicon TC 3E system, using UM 10 membranes, and then dialyzed against several changes of distilled water and lyophilized.

Assay for exfoliatin. Culture supernatants or purified toxin preparations were assayed in newborn mice as previously described (10). Dilutions of samples were made in 10 mM phosphate-buffered saline (0.85%) at pH 7.1.

Assay for hemolysin. Samples of crude culture supernatant or partially purified toxin were tested for hemolytic activity by diluting in 0.15 M NaCl in a microtiter plate and then mixing with a 2% suspension of washed rabbit erythrocytes. The hemolytic titer was expressed as the reciprocal of the highest dilution causing complete lysis of erythrocytes after 30 min at 37°C.

Chromatography. Carboxymethyl (CM)-cellulose (Whatman CM 52) was equilibrated before use in 10 mM phosphate buffer, pH 6.0. Hydroxylapatite (Bio-Rad) was equilibrated in 30 mM phosphate buffer, pH 5.7.

Gel electrophoresis. Polyacrylamide gels (7.5%) in 0.1% sodium dodecyl sulfate (SDS) were prepared by the method of Weber and Osborn (24). Slab gels were prepared by the method of Laemmli (15). Protein

samples were denatured by heating to 100°C for 10 min in a solution of 1% SDS and 0.1% β-mercaptoethanol. Gels were stained with Coomassie brilliant blue and destained by diffusion.

Antisera. Antisera were produced in rabbits by repeated injections of purified exfoliatin. The first injection was mixed with Freund complete adjuvant and given intramuscularly. Subsequent injections were given with toxin alone. A total of 6 mg of toxin, given over 2 months, was used to obtain antiserum for use in Ouchterlony diffusion tests.

Amino acid analyses. Samples of purified toxin in 6 N HCl were hydrolyzed in sealed tubes in vacuo at 110°C for 24 h. Analyses were carried out on a Beckman 121-MB amino acid analyzer interfaced with Beckman system AA computing integrator. Threonine and serine were corrected for destruction during hydrolysis using the factors determined by Rees (18). Tryptophan was determined spectrophotometrically by the method of Edelhoch (5). Three preparations of each toxin were analyzed; each sample was run in triplicate.

C-terminal amino acid determination. Carboxypeptidases A and B were used to cleave C-terminal amino acids, following the procedure of Guidotti (7). Incubations were done at 37°C by using a substrate-enzyme ratio of 20:1.

Amino acid sequence determination. Intact protein (100 nmol) from *S. aureus* TA and DI was subjected to automated degradation on a Beckman 890 C sequencer. Three runs were performed on each toxin. The Beckman standard fast Quadrol protein program proved superior to the dimethylallylamine peptide program for both proteins. Amino acid residues were identified directly from the phenythiohydantoin (PTH) derivatives by gas chromatography (8) or by thin-layer chromatography using the ascending chromatographic method of Jeppsson and Sjöquist (9) and the gradient elution system of Tarr (23). Acid hydrolysis of the PTH derivatives, followed by identification on a Beckman 121-MB amino-acid analyzer of the products, was the final method of residue identification (22).

Histological examination. Sections of epidermis, as well as other organs, from control and toxin-injected mice were examined by light microscopy. Tissues were embedded in paraffin, sectioned (6 μm thick), and stained with hematoxylin and eosin.

RESULTS

Purification of toxin. Portions of lyophilized, dialyzed culture supernatant from strain DI were purified by column chromatography. CM-cellulose was equilibrated at pH 6.0 in 10 mM sodium phosphate. A column (2.5 by 40 cm) was filled with resin and washed with the equilibration buffer; the sample was then run onto the column at a rate not exceeding 1 ml/min. After the entire toxin sample had been applied, the column was washed with several column volumes of buffer, until the absorbance of the eluate had returned to base line. No toxic activity could be detected in the unadsorbed material. The toxin was eluted from the column by increasing the molarity of the buffer to 50 mM at pH 6.8. A single peak was obtained, but all fractions in the peak were positive for hemolytic activity, in addition to their exfoliative activity.

To separate the exfoliatin from the alpha-hemolysin, this partially purified material was dialyzed against 30 mM phosphate buffer at pH 5.7. This sample was then applied to a column of hydroxylapatite previously equilibrated with the same buffer. Both exfoliatin and hemolysin bound to the column and were eluted separately with a linear gradient of sodium phosphate buffer, increasing in molarity from 30 to 400 mM. The exfoliatin eluted first (at about 250 mM), and the alpha-hemolysin followed.

Using the conditions described above, culture supernatants from strain DI usually contained 10 to 15 μg of toxin per ml of culture supernatant. Approximately 10 to 12 g of lyophilized crude supernatant were obtained from each 50-liter fermentation. Recovery was about 70% from the CM-cellulose column elution. The hydroxylapatite column yields were somewhat less, usually averaging 40 to 50% recovery. Starting with 2 g of lyophilized crude supernatant material, the average yield of purified toxin ranged from 20 to 35 mg.

Analysis of the peak fractions from the hydroxylapatite column indicated no contaminating hemolytic activity in the exfoliatin preparation. Analysis of purity by SDS gels indicated that the toxin preparation was more than 95% pure. Trace amounts of lower-molecular-weight components were observed in some preparations. These contaminants could be removed by rechromatography of the toxin on CM-cellulose; a linear gradient (10 to 50 mM phosphate) was utilized to elute the toxin.

These purification steps were essentially the same for both types of exfoliatin. However, minor differences in binding efficiency were demonstrated when a mixture of both types of exfoliatin was applied to a column of hydroxylapatite which had been equilibrated as described above. By using a linear gradient of phosphate buffer, the TA-type exfoliatin eluted slightly ahead of the DI-type toxin (Fig. 1). The presence of one or both types of toxin was confirmed serologically by testing each fraction with specific antiserum in Ouchterlony diffusion tests.

Specific activity. Using the newborn mouse model (17), we found the median effective dose of the DI toxin to be 0.5 μg. Thus, both forms of exfoliatin have the same activity (10).

Molecular-weight determination. In SDS-containing polyacrylamide gels, we estimated that the DI-type exfoliatin had a molecular weight of about 26,000, by comparison with known standards. This is very close to the pre-

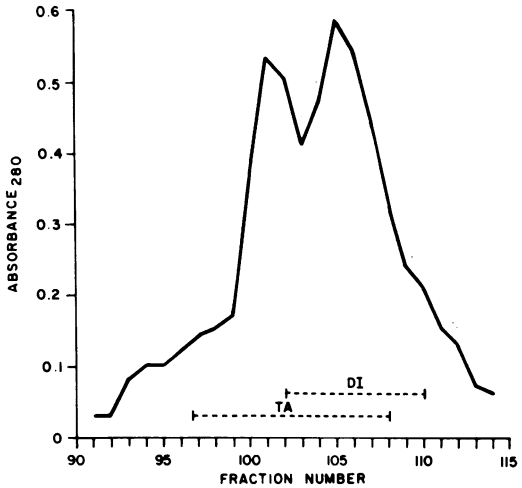

FIG. 1. *Hydroxylapatite chromatography of two forms of exfoliative toxin. A mixture of 10 mg of each of TA-type and DI-type exfoliatins was adsorbed to a column (1.5 by 30 cm) equilibrated with 30 mM phosphate buffer at pH 5.7. A linear gradient of increasing phosphate buffer from 30 to 400 mM was used to separate the two toxins. Dashed lines indicate fractions that reacted with specific antiserum in Ouchterlony diffusion tests.*

viously reported data for TA-type toxin (10), which has a molecular weight of 26,500. In agreement with Wiley and Rogolsky (26), we were unable to separate the two types in conventional cylindrical gels. However, when mixed samples were run in 9% SDS-slab gels, we obtained resolution of the two types. When compared with the individually run cylindrical gels, data confirmed our observation that DI-type toxin migrated slightly ahead of the TA type, and is thus presumably somewhat smaller in molecular weight.

Amino acid composition. The calculated amino acid composition of both types of exfoliatin is presented in Table 1. The error minimization method of Katz (12) gave a single minimum with the TA toxin and was used to obtain the listed values. There are 237 amino acid residues in the molecule corresponding to a molecular weight of 26,600. This analysis is very close to the one we reported previously (10). Usable error plots versus molecular weight were, however, not obtained with DI toxin. Based on the assumption that DI toxin is smaller than the TA toxin and that a molecular-weight difference of 10% would have permitted resolution of the two forms of exfoliatin in cylindrical polyacrylamide gels, the composition of DI toxin was calculated by an iterative process. The molecule has 228 residues and a molecular weight of 25,350. As might be predicted the amino acid composition of both toxins is similar. Neither contains cysteine; both have one residue of methionine. Significant differences were seen in some of the amino acids, notably arginine, alanine, and valine.

Incubation of both types of exfoliatin in the presence of carboxypeptidase-B revealed that the C-terminal amino acid for both types of toxin was lysine. We found that the N-terminal amino acid for the TA-type exfoliatin was glutamic acid, and that for the DI type was lysine.

Amino terminal sequence. The amino acid sequence was obtained for the first 26 and 23 residues of the toxins from strains DI and TA, respectively. A direct comparison of these sequences showed little obvious homology. However, a one residue shift of the TA toxin yielded the alignment shown in Table 2. Eight of the residues (boxed) of the toxin from TA are now identical to those in the DI toxin. Moreover, eight other positions (boldfaced) have amino acids whose codons differ by only a single nucleotide change.

Antiserum reactions. Purified toxin preparations were used to prepare specific antiserum in rabbits to each type of exfoliatin. When reacted in Ouchterlony double diffusion, each antiserum formed a single line of precipitate with 1 μg of homologous antigen, indicating high specificity. However, when sufficiently high concentrations (20 μg) of heterologous toxin were reacted, each antiserum also formed a single line of precipitate with the antigens. When reacted simultaneously, the lines of precipitate crossed, showing complete nonidentity (Fig. 2). Complementary observations to those shown in Fig. 2

TABLE 1. *Amino acid composition of two forms of exfoliatin*

Amino acid	Residues/molecule of exfoliatin	
	TA chromosomal	DI plasmid
Lys	22	22
His	7	5
Arg	9	5
Asp	34	29
Thr	12	12
Ser	17	17
Glu	27	26
Pro	10	12
Gly	24	21
Ala	8	13
½-Cys	0	0
Val	13	9
Met	1	1
Leu	15	16
Ile	17	17
Tyr	11	13
Phe	9	9
Trp	1	1

TABLE 2. *Comparisons of the amino terminal sequences of toxins TA and DI*[a]

Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
DI	Lys	Glu 1	Tyr	**Ala**	Ala	Glu 5	Glu	Ile	**Arg**	Lys	**Leu** 10	**Lys**	Glu	Lys 13	Phe	**Glu**	Val	Pro	Pro	Thr	**Asp** 20	Lys	**Glu**	**Leu** 23	Tyr	Thr
TA		Glu	Val	**Thr**	Ala	Glu	Glu	Ile	**Lys**	Lys	**His**	**Glu**	Glu	Lys	Trp	**Asp**	Lys	Tyr	Tyr	Gly	**Val**	X	**Asx**	**Phe**		

[a] A single unit shift produces the indicated homology. Identical residues are boxed and residues whose codons differ by one nucleotide are in boldface.

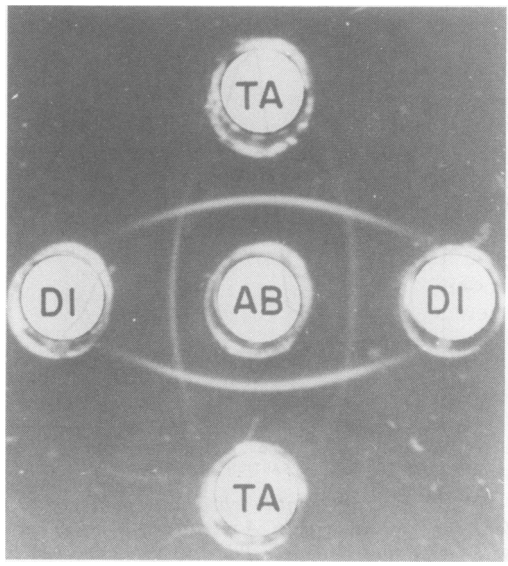

FIG. 2. *Double immunodiffusion of two forms of exfoliative toxin with antiserum to TA toxin. Wells labeled TA contain 1 μg of TA toxin. Wells labeled DI contain 20 μg of DI toxin. The center well contains 5 μl of anti-TA serum. The plate was incubated for 24 h at room temperature.*

for anti-TA toxin were obtained with antisera to DI toxin. These reactions could be completely eliminated by absorbing the serum with the appropriate heterologous toxin. The presence of two distinct antibody populations in each serum seemed to indicate that the antigens used for immunization were not as highly purified as desired. The trace amounts of secondary antigens were apparently present in sufficient quantity to give rise to precipitating antibodies.

Pathological findings. Control mice injected with normal saline showed no pathological lesions. Animals injected with either the TA or DI exfoliatin were typified by multiple areas of separation within the stratum granulosum accompanied by subsequent elevation of the superficial layers of epidermis. These findings were first evident at 2 h after injection of 1 μg of either form of toxin. No other lesions were observed in other organs that were examined, including those from the digestive, respiratory, urinary, cardiovascular, and central nervous systems.

DISCUSSION

Our earlier work described purification of one serological form of exfoliative toxin from strain TA of *S. aureus* (10). We attempted to eliminate toxin production in this strain by specific treatments to cure plasmids (e.g., growth at elevated temperature). Since we were unsuccessful in the

experiments, we can presume chromosomal sites for synthesis of this form of toxin, agreeing with Rogolsky et al. (20). We have now used strain DI for production of a serologically and chemically distinct molecular species of exfoliative toxin. Rogolsky et al. (21) previously studied toxin production by this culture and indicated that in this instance the controlling gene(s) are located on a 56S plasmid. Although we have referred to this form of toxin as plasmid form, preliminary curing experiments indicate that there may be an additional chromosomal site for its synthesis.

There have been several reports dealing with serological differences among types of staphylococcal exfoliatin. Kondo et al. reported the isolation of a toxin from several strains of phage group I staphylococci and has termed this product exfoliatin B to distinguish it from the toxin produced by group II staphylococcal strains, which he termed exfoliatin A (13). Arbuthnott and Billcliffe identified two serological forms of exfoliatin (both from strains of phage group II staphylococci) by using a radial diffusion procedure (1). Others have also described serological differences in exfoliating materials derived from several strains (4, 6, 11). More recently, Wiley and Rogolsky (26) serologically differentiated two exfoliating toxins synthesized under chromosomal and plasmid control.

Discrepancies have also been reported on the stability and molecular weight of some of these toxin preparations (13, 14, 25). The amino acid composition of both types of exfoliatin isolated by Kondo et al. (14) provided clear proof that his toxins were quite different from the two we isolated. Indeed, it may be that each toxin-producing strain of *S. aureus* elaborates a distinct type of exfoliatin, with the products differing in both their serological reactions and chemical composition. Thus far the only property that appears to be relatively consistent is the specific biological activity, suggesting at least some homology among the various toxic molecules. We have demonstrated here significant homology in the amino-terminal sequences of the TA and DI toxins, and one would anticipate that these two molecules have similar secondary and tertiary folding. The toxin from strain TA has been crystallized, and preliminary X-ray diffraction patterns have been obtained (27). Establishment of its three-dimensional structure will be of great value in elucidation of the mechanism of exfoliation.

The two forms of toxin purified in our laboratory have identical specific activities when assayed in the newborn mouse model. We have shown here that these two purified preparations are serologically distinct. We have additionally demonstrated that there are no common antigenic determinants (A. D. Johnson, L. Spero, and J. F. Metzger, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, B99, p. 30) by antigen-binding capacity measurements on radiolabeled toxins. Further studies concerning the genetic control of the production of chemically and serologically different forms of exfoliative toxin are under active investigation in our laboratory.

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