Comparison of Purified Alpha-Toxins from Various Strains of *Staphylococcus aureus*

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Alpha-toxin from five strains of *Staphylococcus aureus*, including Wood 46, was purified by isoelectric focusing. The alpha-toxins obtained from different strains were similar. The isoelectric point of the purified toxins was 8.65 ± 0.15. Sharp concentration peaks were not always obtained. In the ultracentrifuge the alpha-toxins migrated usually as three peaks which could be dissociated with propionic acid to yield one peak. A single line of identity was obtained in immunoelectrophoresis when a heterologous antiserum was reacted with the five purified toxins. It was concluded that the widespread use of the Wood 46 strain for the production of alpha-toxin is justified.

*Staphylococcus aureus* Wood 46 is the strain used by most investigators in studies of staphylococcal alpha-toxin. Recent reports (1, 2, 9, 11) have indicated certain dissimilarities in the alpha-toxins purified in different laboratories. This fact, together with the widespread interest in alpha-toxin, led us to examine five different toxigenic strains of *S. aureus* to determine whether the toxins produced were identical with respect to physical and immunological characteristics and whether the use of *S. aureus* Wood 46 as the principal source of alpha-toxin was justified.

**MATERIALS AND METHODS**

**Bacterial strains.** The Wood 46 strain of *S. aureus* was obtained from R. Altenbern. Sixty strains of *S. aureus* were obtained from the Center for Disease Control (CDC), Atlanta, Ga. Four of the CDC strains that produced the greatest amounts of alpha-toxin were selected for study. Subcultures for testing were obtained from well-isolated colonies showing large zones of hemolysis on rabbit blood agar. All five of the strains had distinctly different phage types when tested by 22 phages of the International Typing Series.

**Media and growth conditions.** The media and growth conditions have been described previously (5). Trypticase soy broth (Baltimore Biological Laboratories) containing 0.3% Difco yeast extract (TSY) in 400-ml quantities in 12-liter flasks was inoculated with 5-ml quantities of an overnight TSY broth culture and incubated at 37 C with shaking for 20 h.

**Purification of toxin.** The purification procedure for staphylococcal alpha-toxin has been described (5). Proteins were precipitated by saturation of concentrated culture supernate to 66% with ammonium sulfate. The proteins were subjected to wide-range (pH 3 to 10) and narrow-range (pH 7 to 10) isoelectric focusing. Protein concentrations were determined by the method of Lowry et al. (6) by using bovine serum albumin as a standard. Ultracentrifugation of purified alpha-toxin samples was done in a Beckman model E analytical ultracentrifuge equipped with schlieren optics. Immunoelectrophoresis of the purified products was as previously described (5).

**Kinetic studies.** The rates of hemolysis of rabbit erythrocytes by purified alpha-toxin were measured by using a turbidimetric method similar to that of Wright (13). The transmission at 650 nm was continuously monitored in a Beckman spectrophotometer. The reference cell contained 3 ml of PBSG buffer (0.2 M monosodium phosphate, 45 ml; 0.2 M disodium phosphate, 55 ml; sodium chloride, 0.85% [wt/vol]; gelatin, 0.1% [wt/vol]; water, to 200 ml; pH 6.9). Reaction mixtures consisted of 2 ml of PBSG, 0.5 ml of the toxin sample, and 0.5 ml of a 0.2% suspension of rabbit erythrocytes (absorbance of 0.6). The toxin sample which was kept at 0 C was added last. The other reagents were kept at 20 C. The temperature in the cuvette compartment was maintained at 20 C.

**RESULTS**

**Narrow-range electrofocusing of alpha-toxins.** The Wood 46 strain consistently produced the largest amounts of alpha-toxin. Hemolysin titers of 2.5 × 10^8 hemolytic units (HU) per ml of culture supernate were obtained. The hemolysin titers of the other four strains ranged from 4 × 10^7 HU per ml to 8 × 10^8 HU per ml.

The results obtained by electrofocusing Stage 5 (5) alpha-toxins in a narrow-range (pH 7 to pH 10) gradient are shown in Fig. 1. The toxin...
from Wood 46 concentrated maximally at about pH 8.60 with shoulders of hemolytic activity on either side of the peak fraction (Fig. 1A). Stage 5 alpha-toxin, from strain 57, yielded maximum hemolytic activity at pH 8.55 (Fig. 1B). A small shoulder of activity occurred on the basic side of the curve. For Stage 5 alpha-toxin from strain 3079 maximum hemolytic activity occurred at pH 8.80 (Fig. 1C). A shoulder of hemolytic activity occurred on the acidic side of the curve. Hemolytic activity of Stage 5 alpha-toxin from strain 3558 was maximal at pH 8.65 but was broadly distributed (Fig. 1D). In addition, hemolysis of horse erythrocytes, a measure of delta hemolysin activity, occurred in fractions of pH 9.15 to pH 9.45. This strain produced several major proteins which focused in this basic pH range. For Stage 5 alpha-toxin from strain 3565 maximum alpha-hemolytic activity focused at pH 8.45 (Fig. 1E). Delta-hemolysin activity was detected in the more basic (pH 9.10 to pH 10.20) fractions.

**Analytical ultracentrifugation of Stage 7 alpha-toxins.** Schlieren patterns obtained when Stage 7 (5) alpha-toxin was analyzed by ultracentrifugation revealed two peaks, with sedimentation coefficients of 10.5 and 3.0S for the large and small components, respectively. An exception to this pattern was strain 57 alpha-toxin which gave but a single 3.0S peak. After dialysis against 1 M propionic acid and recentrifugation, alpha-toxins from each of the other strains gave a schlieren pattern similar to that of strain 57.

**Immunoelectrophoresis of Stage 7 alpha-toxins.** The results of immunoelectrophoresis of Stage 7 alpha-toxins is shown in Fig. 2. The central wells contained antiserum prepared against the crude toxin of strain Wood 46. The peripheral wells contained Stage 7 alpha-toxin from the various strains. The reaction between antitoxin and crude alpha-toxin yielded at least seven distinct precipitin lines, but only one line was obtained from the reaction between the purified toxins and the antitoxin. Antisera to Stage 7 toxin from each of the 5 strains of *S. aureus* gave single precipitin lines with both Stage 7 toxins and crude toxins. In addition, a single line of identity was obtained with all five of the purified toxin samples by double diffusion in agar.

103.0 mg of protein focused; C, strain 2079, 212.2 mg of protein focused; D, strain 3558, 287.0 mg of protein focused; E, strain 3565, 150.0 mg of protein focused. Symbols: ○, pH; ●, absorbance at 280 nm; Δ, hemolytic units (rabbit blood); ▲, hemolytic units (horse blood).
Fig. 2. Immunoelectrophoretic analysis of purified alpha-toxin samples. The central well in each plate contained crude alpha-toxin; each trough contained antiserum prepared against crude toxin. The outer wells contained purified alpha-toxin from strains Wood 46 (well 1), 57 (well 2), 3079 (well 3), 3558 (well 4), Wood 46 (well 5), and 3565 (well 6). The cathode was at the right.
Kinetic studies. When the percentage hemolysis of rabbit erythrocytes by various concentrations of the purified alpha-toxin samples was plotted against the time, the sigmoidal curves obtained were typical for alpha toxin (8, 13). The only variation noted was with alpha toxin purified from strain 3079. This alpha toxin sample had low activity at 20°C. When assayed at 37°C its activity was essentially equivalent to the activities of the other toxin samples at 20°C.

DISCUSSION

The almost exclusive use of the Wood 46 strain of S. aureus for the study of alpha-toxin and production of antitoxin raised the question of whether alpha-toxins obtained from different strains were in fact identical. Wood 46 produced the largest zone of hemolysis on rabbit blood agar and also gave the highest hemolysin titers when grown in broth culture. Since the other organisms used were selected because they were the best toxin producers of more than sixty strains tested, the widespread use of Wood 46 to obtain high yields of alpha-toxin seems justified. However, since the specific activity of purified Wood 46 alpha-toxin was two to five times greater than the specific activities of purified toxins of the other strains, it is possible that the differences in hemolysin titers resulted in part from fundamental differences in the toxins themselves. The specific activity (18,000) of the Wood 46 alpha-toxin obtained in this study is in good agreement with the results of other workers (2, 3) and the specific activities of the other purified toxins are within the range of reported values (1).

An analysis of the kinetic curves of the purified alpha-toxin samples revealed the typical sigmoidal shapes obtained by other workers (8, 13). The reaction rates varied with the concentration of alpha-toxin (in hemolytic units) but were similar for the toxins from different strains. The minor variations seen would be expected because of dilution and other titration errors. The trailing off in rate of hemolysis observed at low toxin concentration was probably due to heat lability of the toxin (7), and it was to minimize this inactivation that 20°C was chosen as the reaction temperature. However, 20°C seemed to inhibit the hemolytic action of the alpha-toxin of strain 3079, while at 37°C rates of hemolysis similar to those of the other toxin samples were obtained.

The isoelectric point of the purified toxins was 8.65 ± 0.15, but sharp concentration peaks were not generally obtained. These values are within those reported in the literature (9, 10). The spread in values determined for the isoelectric points probably owed to the large amount of protein within the focused zone, as well as to the heterogeneity in charge (12) of individual toxin molecules. In contrast to the findings of Molloy and Wadstrom (10), but in agreement with McNivin et al. (9), refocusing in the more narrow-range pH gradient did not yield multiple forms of alpha-toxin.

When purified alpha-toxin is analyzed in the ultracentrifuge, the schlieren patterns often reveal multiple peaks (1, 2, 4). Similar patterns were obtained using the alpha-toxins in this study. It has also been reported (1) that such samples contain alpha-toxin multimers which can be dissociated to yield the monomeric toxin. That this was the case in the present instance was shown by dissociation of the samples in propionic acid and recentrifugation. In each case single peaks were obtained.

Most of the purified toxin samples were stored in the frozen state prior to ultracentrifugal analysis. Alpha-toxin from strain 57, however, was analyzed immediately after the final purification step. The schlieren pattern for this toxin revealed only a single peak before dissociation. In light of Coulter's (3) observation of the rapid inactivation of stored alpha-toxin and of Bernheimer's (2) results showing little activity associated with his 12S alpha-toxin, it is tempting to speculate that, in each instance, only one alpha-toxin peak would have been found had centrifugation been performed before the toxins were frozen.

The results from immunoelectrophoresis and double diffusion in agar indicate that alpha-toxins obtained from these strains are immunologically identical. The similarities in the migration patterns in the ultracentrifuge and in the kinetic behavior of the toxins are also indications of identity. The minor variations, such as the ultracentrifugational pattern of strain 57 alpha-toxin and the effect of temperature on the kinetics of strain 3079, demand further investigation before their precise significance can be stated. Also, the higher specific activity of strain Wood 46 alpha-toxin can be explained only when additional data, such as amino acid analyses of each toxin, are available. It seems probable that most of these variations are of a minor nature. Since the alpha-toxins produced by distinctively different strains of S. aureus are immunologically identical, the continued use of strain Wood 46 for studies on alpha-toxin seems justified.
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LITERATURE CITED

1. Arbuthnott, J. P. 1970. Staphylococcal α-toxin, p. 189-236. In S. J. Aji, S. Kadis, and T. C. Montie (ed.), Microbial toxins, vol. 3. Academic Press Inc., New York.
2. Bernheimer, A. W. 1968. Cytolytic toxins of bacterial origin. Science 159:847-851.
3. Coulter, J. R. 1966. Production, purification, and composition of staphylococcal α-toxin. J. Bacteriol. 92:1655-1662.
4. Forlani, L., A. W. Bernheimer, and E. Chiancone. 1971. Ultracentrifugal analysis of staphylococcal alpha toxin. J. Bacteriol. 106:138-142.
5. Goode, R. L., and J. N. Baldwin. 1973. Purification of staphylococcal alpha toxin by electrofocusing. Prep. Biochem. 3:349-361.
6. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.
7. Marucci, A. M. 1963. Mechanism of action of staphylococcal alpha-hemolysin. I. Same factors influencing the measurement of alpha-hemolysin. J. Bacteriol. 86:1182-1188.
8. Marucci, A. M. 1963. Mechanism of action of staphylococcal alpha-hemolysin. II. Analysis of the kinetic curve and inhibition by specific antibody. J. Bacteriol. 86:1189-1195.
9. McNiven, A. C., P. Owen, and J. P. Arbuthnott. 1972. Multiple forms of staphylococcal alpha-toxin. J. Med. Microbiol. 5:113-122.
10. Mollby, R., and T. Wadstrom. 1969. Studies on hemolysins from Staphylococcus aureus by the method of isoelectric focusing. Protides Biol. Fluids Proc. Colloq. 17:446-469.
11. Six, Howard, R., and Sidney Harshman. 1973. Purification and properties of two forms of staphylococcal α-toxin. Biochemistry 12:2672-2677.
12. Wadstrom, T. 1968. Studies on extracellular proteins from Staphylococcus aureus. IV. Separation of α-toxin by isoelectric focusing. Biochem. Biophys. Acta 168:228-242.
13. Wright, M. R. 1969. Effect of pH on the hemolysis of rabbit erythrocytes by staphylococcus alpha hemolysin. J. Med. Microbiol. 2:301-308.