Further Characterization of Staphylococcal Gamma-Hemolysin

ANTHONY G. TAYLOR and ALAN W. BERNHEIMER

Department of Microbiology, New York University School of Medicine, New York, New York 10016

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It was confirmed that staphylococcal gamma-hemolysin is composed of two separate proteins (gamma-lysin components I and II) which act synergistically. The molecular weights of the two components, determined by gel filtration, are 29,000 and 26,000, respectively, and their isoelectric points, determined by isoelectric focusing, are at pH 9.8 and 9.9. Both components are susceptible to the action of Pronase and subtilisin. A wide range of lipids, some in minute amounts, are capable of inhibiting the hemolytic activity of gamma-lysin.

Although staphylococcal gamma-hemolysin was first described 25 years ago (16), relatively little progress was made in its study until the recent work of Plommet and his co-workers in France who described methods for its production, partial purification, and progress in work on Staphylococcus aureus (10). It was shown to have two components (here referred to as gamma-lysin component I and gamma-lysin component II) which act synergistically, both being necessary not only for hemolysis, but also for toxicity in mice (6). The development of specific neutralizing antibodies to the hemolysin in human staphylococcal bone disease has been reported (18), and these results have also indicated that a high proportion of Staphylococcus aureus strains involved in such disease processes produce this toxin in vivo. The existence and possible importance of gamma-hemolysin or gamma-toxin is therefore no longer in doubt.

In addition to confirming the general findings of Plommet et al., we report further physicochemical properties of the hemolysin. The demonstration of a number of inhibitors readily permits it to be distinguished from the well-characterized staphylococcal hemolysins.

MATERIALS AND METHODS

Measurement of hemolytic activity. Gamma-hemolysin was assayed by the method of Guyonnet and Plommet (7). Unless otherwise stated, isotonic phosphate-buffered saline (PBS; pH 6.8) was used as the diluent and prepared as described by Jackson and Little (10). It contained (per liter): KH2PO4, 1.029 g; NaH2PO4, 12 H2O, 4.02 g; and NaCl, 8.0 g. One hemolytic unit (HU) is that amount of lysin required to produce 50% lysis of a standardized human erythrocyte suspension. That suspension (ca. 12% vol/vol) when lysed by addition of 48 volumes of 0.1% sodium carbonate gives an optical density of 0.5 at 540 nm. The assay of gamma-lysin components I and II was performed individually under identical conditions but with the addition of a mild excess (50 HU) of the absent component to each tube in the assay series.

Gamma-hemolysin production. Gamma-hemolysin was prepared by the method of Bézard and Plommet (2) using S. aureus strain 5R described by Smith (15). Briefly, the method was as follows. The lyophilized organism was suspended in broth, cultured at 37°C for 4 h, then transferred to human blood agar (Lonagar, Oxoid, London) plates, and incubated for 24 h in an atmosphere of 80% oxygen and 20% carbon dioxide. Rough hemolytic colonies were then selected for repassage on fresh plates. This process was repeated four times. Colonies were then harvested, transferred to nutrient agar slants (Difco heart infusion broth and Lonagar), and incubated overnight. The resulting colonies were suitable for use as seed in the primary liquid medium, or, alternatively, the slants could be stored at 4°C for up to 1 month. The culture medium is a modification of the casein hydrolysate-yeast extract medium of Gladstone and van Heyningen (5). We first treated the yeast dialysate, however, by batch adsorption with hydroxyapatite (Bio-Rad Laboratories, New York) in 0.001 M sodium phosphate (pH 8.6). At this low molarity much pigmented material is adsorbed, but the supernate is well able to support toxin production.

The organisms harvested from one slant were seeded into 50 ml of medium in a 250-ml Erlenmeyer flask and incubated at 37°C with agitation for 4 h. A 10-ml portion of this culture was transferred to another 100 ml of medium for 4 h and this was then used to inoculate 10 liters of medium in a 20-liter fermenter. The starting pH of 6.8 was maintained near neutrality by the automatic addition of sodium hydroxide. Aeration was achieved by the passage of 80% oxygen and 20% carbon dioxide at a rate of 1 liter/min. After 16 h the culture normally contained...
600 to 1,300 HU/ml, and the specific activity was approximately 40 HU/mg of protein. The bacteria were removed by continuous-flow centrifugation of the supernatant after they were allowed to sediment for 3 to 5 h at 4°C.

**Purification of gamma-hemolysin and separation of gamma-lysin components I and II.** The gamma-hemolysin was purified as previously described (2, 7). Initially, the ionic strength of the culture supernatant was lowered by a 1:3 dilution with distilled water, under which conditions both toxin components are adsorbed by hydroxyapatite, which was added as a suspension in 0.001 M sodium phosphate (pH 6.8) using 1 g of adsorbent per 10,000 HU. The hydroxyapatite was washed three times with 0.2 M sodium phosphate (pH 6.8) to remove weakly binding impurities; the two toxin components were then eluted together by the addition of 0.7 M sodium phosphate (pH 6.8). The crude toxin was concentrated on Diaflo PM 10 membranes (Amicon Corp., Lexington, Mass.) and then dialyzed against 0.1 M sodium phosphate buffer (pH 6.8). The volume at this stage was about 150 ml and the total activity was approximately 5 × 10^6 to 10 × 10^6 HU. The specific activity was about 1,500 HU/mg. The toxin was then purified further by column chromatography on hydroxyapatite. Adsorption was in 0.1 M sodium phosphate, washing was with 0.2 M, elution of gamma-lysin component I was with 0.3 M, and elution of gamma-lysin component II was with 0.5 M sodium phosphate, all manipulations being at pH 6.8. The specific activity after dialysis against distilled water and mixing of the two fractions was about 4,000 HU/mg, and the final yield from a 10-liter culture was 2.5 × 10^9 to 5 × 10^9 HU. The overall purification of the hemolysin in the culture supernatant was approximately 100-fold.

**Isoelectric focusing.** Isoelectric focusing experiments were performed on a 110-ml column (LKB Instruments Inc., Rockville, Md.) as described by Vesterberg et al. (19). The conditions used for the pH 3 to 10 gradient were those described by Taylor (17). Isoelectric focusing in a basic pH gradient with pH 9 to 11 Ampholine (LKB Instruments Inc., Rockville, Md.) was as described by Karlsson and Ohman (12) for cytochrome c, except that 85% glycerol was used as the heavy component of the stabilizing density gradient in place of sorbitol. Measurements of pH were made at 4°C with an Orion Research Model 701 pH meter with a pH 0 to 12 glass electrode (A. H. Thomas, Philadelphia, Pa.).

**Proteases.** Pronase (grade B) was from Calbiochem, Los Angeles, Calif., and subtilisin (type VII) was from Sigma Chemical Co., St. Louis, Mo. The potency of these enzymes was determined by using casein as the substrate. The enzyme (2 ml) in 0.2 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.4) was incubated at 37°C for 30 min with 1 ml of dialyzed casein (4%). The reaction was stopped by the addition of 2 ml of trichloroacetic acid (10%) and the mixture was filtered. The absorption at 280 nm was then measured in a Zeiss spectrophotometer. Pronase (100 μg/ml) produced an E_280/1 cm of 1.5 in the above assay, and subtilisin at the same concentration produced an E_280/1 cm of 2.6.

**Lipids.** Lecithin (preparation 1) and sphingomyelin were from General Biochemicals, Chagrin Falls, Ohio. Lecithin (preparation 2), stearic acid, palmitic acid, and nervonic acid were from Applied Science Laboratories, Inc., State College, Pa. Phosphatidyl ethanolamine and phosphatidyl serine were from Supelco Inc., Bellefonte, Pa. Diphosphatidyl glycerol (cardiolipin) was from Sylvana Chemical Co., Milburn, N.J. Phosphatidyl inositol and phosphatidyl glycerol from Micrococcus lysodeikticus were a gift from Peter Owen. Cholesterol, cholesterol acetate, and cholesterol were from Sigma Chemical Co., St. Louis, Mo.

**Preparation of phospholipid suspensions.** A chloroform solution of the lipid (5 to 25 mg/ml) was pipetted into a boiling tube (150 by 21 mm). The solvent was evaporated under a stream of nitrogen, leaving the lipid as a thin film over the lower part of the tube. After addition of PBS, the lipid was suspended by vigorous agitation on a Vortex mixer, giving a final lipid concentration of 2 mg/ml from which the desired dilutions were made.

**Preparation of fatty acid solutions.** The fatty acids were dissolved in ethanol and then diluted in PBS. The concentration of ethanol in the working dilutions never exceeded 2%, and in all cases control experiments with ethanol but without fatty acids were included in experiments.

**Preparation of solutions of cholesterol, cholesteryl acetate, and cholestanol.** The lipid was dissolved in glacial acetic acid to give a stock solution (200 mg/ml). This solution was diluted as required in PBS, adjusted to pH 6.8 with sodium hydroxide solution, and made up to volume with PBS.

**RESULTS**

**Isoelectric focusing.** Preparations of gamma-lysin components I and II, separated by hydroxyapatite chromatography, were subjected to isoelectric focusing to determine the isoelectric points of the two proteins. Either gamma-lysin component I or II (5 mg, 15,000 HU) was added throughout the gradient. Runs on a pH 3 to 10 gradient established the highly basic nature of the two components which were both focused in the pH range 9.0 to 10.5. A precise determination of their isoelectric points was not possible in this pH gradient. On a basic pH gradient with pH 9 to 11 Ampholine, however, the isoelectric points of gamma-lysin component I was found to be 9.8 ± 0.2, and that of gamma-lysin component II was found to be 9.9 ± 0.2 (Fig. 1 and 2). The recovery of hemolytic activity in each case was about 40%.

**Estimation of molecular weights of gamma-lysin components I and II by gel filtration.** A G-50 Sephadex (Pharmacia Fine Chemicals, Piscataway, N.J.) column (90 by 2.5 cm diameter) was equilibrated with 0.5 M sodium phosphate (pH 6.8) buffer. The column was calibrated as described by Andrews (1) us-
ing the following marker proteins: crystalline bovine serum albumin, crystalline bovine pancreatic ribonuclease (Calbiochem, Los Angeles, Calif.); cytochrome c, chymotrypsinogen A (Sigma Chemical Co., St. Louis, Mo.); beta-lactoglobulin (gift of D. Levieux). Gamma-lysin components I and II when mixed in equal amounts had a specific activity of 3,000 HU/mg. Marker protein (5 mg) was applied to the column in 2 ml of buffer and located by measuring the absorption of the fractions (3 ml) collected at 280 nm. Gamma-lysin components I and II were located by titration of the hemolytic activity of the fractions after addition of the complementary component. The molecular weights of gamma-lysin components I and II were estimated to be 29,000 and 26,000, respectively (Fig. 3). Similar results were obtained by using a thin-layer gel filtration technique.

A question arising from the observation that gamma-lysin has two components is whether these components are separate entities or could possibly be subunits arising during chromatography and other manipulations. When crude culture filtrate after dialysis against the buffer (0.5 M sodium phosphate, pH 6.8) was applied to the column, the hemolytic activity emerged in the same elution volume as the separate toxin components. This suggests that the two components are present as such in the culture supernatant.

**Behavior of gamma-lysin on DEAE-Sephadex and carboxymethyl-cellulose.** The behavior of gamma-lysin on these adsorbents was studied in view of its anomalous behavior reported by Möllby and Wadström (14). A culture supernatant (600 HU/ml) was dialyzed against 0.05 M Tris-hydrochloride (pH 8.5), and 1 ml of this material was applied to a column of diethylaminoethyl (DEAE)-Sephadex A 25 (Pharmacia Fine Chemicals, Piscataway, N.J.; 18 by 0.5 cm, previously equilibrated with the same buffer). Fractions were assayed for gamma-lysin which appeared in the eluate in 100% recovery. Separated gamma-lysin components I and II behaved similarly, confirming that gamma-lysin is not adsorbed to DEAE-groups under these conditions.

Similar experiments were performed with carboxymethyl (CM)-cellulose (Whatman CM 52, H. Reeve Angel Inc., Clifton, N.J.) equilibrated with 0.05 M sodium phosphate (pH 6.5), under which conditions both toxin components were adsorbed. The two fractions were eluted by stepwise increase of sodium chloride molarity to 0.5 M. We have found, therefore, that our gamma-lysin when subjected to ion-exchange chromatography under the conditions described by Möllby and Wadström (14) behaved as expected for basic proteins.

**Effect of proteases on gamma-lysin compo-**
nants I and II. The effect of both Pronase and subtilisin on gamma-lysin components I and II was investigated to gain further evidence for the assumption that the two toxin components are proteins. Gamma-lysin components I and II were each employed at a concentration of 33,000 HU/ml. Incubation of the toxin with increasing concentrations of enzyme was performed in sealed microtiter trays (Cooke Engineering Co., Alexandria, Va.) by using 0.1 ml of enzyme in 0.2 M Tris-hydrochloride (pH 7.4) together with 0.05 ml of gamma-lysin components I or II in the same buffer. At the end of the incubation period (15 or 30 min), the trays were immersed in ice water, and in each case 0.05 ml of the reaction mixture was diluted in 20 ml of cold PBS to minimize further digestion. The hemolytic activity of each sample was titrated after the addition of the complementary toxin component. The percentage of activity remaining after enzyme treatment compared with that in the controls (without enzyme) was plotted against the enzyme concentrations used. The results of experiments with subtilisin are shown in Fig. 4, which shows that gamma-lysin component II is the more susceptible. Similar results were obtained with Pronase. The absorption spectra of the preparations of gamma-lysin components I and II were also typical for proteins.

Inhibitors of gamma-lysin. The effects of various possible inhibitors on gamma-lysin having a specific activity of 3,000 HU/mg were investigated in the standard assay by using the toxin at a concentration of 15 to 30 HU/ml. The inhibitors were incorporated in the PBS to which the toxin was added 10 min before the addition of the erythrocytes. The concentration of inhibitor given is that in the final reaction mixture.

(i) Cholesterol, cholesterol acetate, and cholesteryl. The solubility of these compounds in PBS only allowed the study of inhibition up to concentrations of about 20 \( \mu \)M, since above this concentration precipitation of the lipid occurred. The inhibition curves (Fig. 5) do not show 100% inhibition for this reason. With low concentrations of toxin, complete inhibition can be demonstrated. The inhibition by cholesterol acetate shows that the 3-beta-hydroxyl group is not essential for binding to gamma-lysin.

(ii) Phospholipids and fatty acids. The phospholipids tested for an inhibitory effect on gamma-lysin showed considerable variation in their ability to produce inhibition (Table 1). The most effective inhibitor of those tested was cardiolipin which was chromatographically pure. The fatty acids tested were also inhibitory in low concentration.

(iii) Dextran sulfate and heparin. It has been reported (7, 20) that gamma-lysin is inhibited by agar and some other acid polymers. Total inhibition of gamma-lysin (30 HU/ml) was observed with concentrations of dextran

FIG. 5. Inhibitory effect of cholesterol (●), cholesteryl acetate (▲), and cholesteryl (■) on gamma-hemolysin (30 HU/ml).

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Minimal conc (µg/ml) producing 50% inhibition of 15 HU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lecithin (preparation 1)</td>
<td>100</td>
</tr>
<tr>
<td>Lecithin (preparation 2)</td>
<td>80</td>
</tr>
<tr>
<td>Phosphatidyl ethanolamine</td>
<td>&gt;140</td>
</tr>
<tr>
<td>Phosphatidyl serine</td>
<td>5</td>
</tr>
<tr>
<td>Phosphatidyl inositol</td>
<td>0.15</td>
</tr>
<tr>
<td>Phosphatidyl glycerol</td>
<td>0.25</td>
</tr>
<tr>
<td>Diphosphatidyl glycerol (cardiolipin)</td>
<td>0.05</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>&gt;140</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>3</td>
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<tr>
<td>Palmitic acid</td>
<td>4</td>
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<tr>
<td>Nervonic acid</td>
<td>1</td>
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TABLE 1. Inhibition of gamma-hemolysin by lipids
sulfate exceeding 0.1 μg/ml and with heparin at concentrations greater than 0.05 IU/ml. Dextran, ribonucleic acid, deoxyribonucleic acid, and hyaluronic acid were not inhibitory at concentrations of 0.1 mg/ml.

(iv) Trypan blue. Trypan blue inhibited gamma-lysin (30 HU/ml) completely at concentrations exceeding 4 μg/ml.

**DISCUSSION**

Gamma-hemolysin can be separated by hydroxyapatite chromatography into two components, I and II, which synergistically, though not individually, lyse human, rabbit, and sheep erythrocytes, but not those of horses (7). The loss of activity after incubation with Pronase or subtilisin reported here suggests that both components are proteins.

Recently Möllby and Wadström (14, 20) reported the isoelectric point of gamma-lysin to be at pH 9.5, but they were unable to confirm that the hemolysin has two components. We have confirmed the separation of gamma-lysin components I and II on hydroxyapatite first reported by Guyonnet et al. (8) and shown that the isoelectric points of the two components are extremely close (pH 9.8 and 9.9, respectively). Separation of the two proteins could not be achieved by isoelectric focusing using a broad pH 3 to 10 gradient as used by Möllby and Wadström. We have also shown that the molecular weights of the two components are very similar, and one could predict that separation of the two proteins would be difficult by any of the standard techniques which depend on differences in molecular size or charge. Wadström and Möllby (20) did not specify whether they had tried to separate the two components by hydroxyapatite chromatography as originally described, but it would now appear that this technique is so far the only method available for the preparative separation of gamma-lysin components I and II.

We have found that the two components of gamma-lysin, whether applied together or separately, behaved predictably when subjected to ion-exchange chromatography on DEAE-Sephadex or CM-cellulose. Möllby and Wadström found that their gamma-lysin behaved atypically for a basic protein in being adsorbed to DEAE-Sephadex equilibrated with 0.05 M Tris-hydrochloride (pH 8.5), but not to CM-cellulose in 0.05 M phosphate (pH 6.5). The possibility remains that their lysin is yet another staphylococcal hemolysin, but, as they used strain 5R obtained from the French team, this would seem unlikely, especially as the culture medium was similar and aeration was provided by shaking the cultures.

Our results agree with those of Wadström and Möllby (20) with respect to the inhibition of the lysin by sulfonated polymers such as agar, heparin, and dextran sulfate and not by ribonucleic acid, deoxyribonucleic acid, dextran, and hyaluronic acid. This inhibition is undoubtedly associated with binding of the basic toxin to the acid groups of the polymers.

We demonstrated further that gamma-lysin is inhibited by low concentrations of cholesterol. Streptolysin O and the other related oxygen-labile hemolysins are also inhibited by cholesterol, and there is much evidence to suggest that these lysins are bound to the red cell membrane through the 3-beta-hydroxyl group of cholesterol (9; S. Shany, A. W. Bernheimer, P. S. Grushoff, and K.-S. Kim, Mol. Cell. Biochem., in press). Gamma-lysin, unlike these hemolysins, is also inhibited by cholesterol acetate in which the 3-beta-hydroxyl group is esterified. The mechanism of inhibition of gamma-lysin by cholesterol is therefore different and probably nonspecific.

We have shown that a wide variety of lipids inhibit gamma-lysin (Table 1), and in this respect there is some resemblance to delta-lysin (11, 13). However, delta-lysin is not inhibited by fatty acids, and different phospholipids were inhibitory at similar concentrations. In the case of gamma-lysin, the various phospholipids tested varied considerably in their relative ability to inhibit.

The mode of action of gamma-hemolysin is unknown. It is interesting to note that both components have an isoelectric point similar to that of staphylococcal beta-hemolysin now known to be a sphingomyelinase (4), and also to that of delta-lysin which is believed to act nonenzymatically (13). The possibility that both gamma-lysin components are enzymes has been considered in view of the findings of Colley et al. (3), who have shown that human red cells incubated with phospholipase C from Bacillus cereus and also sphingomyelinase from S. aureus are lysed, although neither of these enzymes produces lysis independently. We have, however, found no phospholipase C activity with egg yolk as the substrate, and neither gamma-lysin component separately, nor together, degraded the major lipids extracted from human or rabbit red cells when examined by two-dimensional silica-paper chromatography.

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