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Mechanism of Hemolysis by Renalin, a CAMP-Like Protein from *Corynebacterium renale*

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Synergistic hemolysis of sheep erythrocytes caused by *Staphylococcus aureus* and *Corynebacterium renale* resulted from the combined action of extracellular staphylococcal sphingomyelinase C and a newly described extracellular agent of *C. renale* (renalin). The affinity of renalin for ceramide was considered to play a key role in causing hemolysis in erythrocytes in which ceramide had been generated through the action of sphingomyelinase C.

When *Corynebacterium renale* and a β-toxin-producing strain of *Staphylococcus aureus* are cross-streaked on sheep blood agar, subsequent incubation results in the development of sectors of complete hemolysis in areas in which the two organisms have grown in proximity to each other (6). Superficially, this effect resembles the CAMP reaction, a term applied to synergistic hemolysis resulting from the proximal growth of *Streptococcus agalactiae* and β-toxin-producing strains of *S. aureus* (4). The mechanism of the CAMP reaction appears to involve the sequential action of staphylococcal sphingomyelinase and a ceramide-binding protein from *S. agalactiae* (2). The aim of this study was to elucidate the mechanism of synergistic hemolysis produced by *C. renale* and *S. aureus*.

Among five strains of *C. renale* that were cross-streaked with *S. aureus* strain C128 on sheep blood agar, four showed synergistic hemolysis and one did not. To test for a soluble extracellular growth product of *C. renale* that might act conjointly with staphylococcal sphingomyelinase C (β-toxin), the same five strains were grown in a yeast-diffusate casein hydrolysate medium (1). The culture supernatants, which were not hemolytic by themselves, were then assayed for capacity to lyse washed sheep erythrocytes in the presence of a small fixed amount of staphylococcal sphingomyelinase C (2). The *C. renale* culture supernatants were diluted in 0.01 M Tris (pH 7.2)–0.145 M NaCl–0.01 M $MgCl_2$–0.2% gelatin (buffer 1), and the conditions of the assay were identical to those described previously for the assay of CAMP activity (2). Activity was expressed as renalin units (RU); 1 RU was defined as the smallest amount of test solution that caused 50% hemolysis. Culture supernatants of the four strains that were positive in the plate test contained 500 to 3,000 RU/ml, whereas the supernatant of the strain that was negative in the plate test showed no activity in the tube assay.

For routine production of "renalin," the active agent in cultures of *C. renale*, 2 ml of a late-log-phase, neopeptone-meat infusion broth culture of *C. renale* strain 8 was added to each of two 2-liter Erlenmeyer flasks, each of which contained 600 ml of yeast-diffusate casein hydrolysate medium (1). The cultures were grown for 48 h in a 37°C shaking water bath at 150 rpm. Under these conditions, a turbidity of 5.5 to 11.0 absorbance units, as measured in a standard Zeiss spectrophotometer with 650-nm light and a 10-mm path, developed. The culture supernatant usually contained 800 to 1,700 RU/ml. The cultures were pooled and centrifuged at 14,500 × *g* for 60 min. Ammonium sulfate (560 g) was dissolved with stirring into approximately 1 liter of supernatant. After standing at room temperature for 1 to 2 h, the precipitate was sedimented by centrifugation at 19,000 × *g* for 15 min. It was dissolved in about 4 ml of 0.03 M sodium borate–0.1 M KCl (pH 8.2; buffer 2), dialyzed against 900 ml of the same buffer, pervaporated to a volume of about 4 ml, and clarified by centrifugation.

The supernatant (4 ml) was fractionated in a refrigerated column of Sephacryl S-200 (Pharmacia Fine Chemicals; 2.5 by 30 cm), equilibrated with buffer 2, and eluted with buffer 2 at a flow rate of 20 ml/h. The fractions (4 ml each) that comprised the peak of activity were pooled, and the pool was dialyzed against 80% saturated ammonium sulfate. The precipitate was separated by centrifugation and then dialyzed against buffer 2. The resulting material was recycled two more times in the Sephacryl column and finally was precipitated with 80% saturated ammonium sulfate. The distribution of renalin activity and absorbance at 280 nm in third-cycle Sephacryl S-200 fractions are shown in Fig. 1.

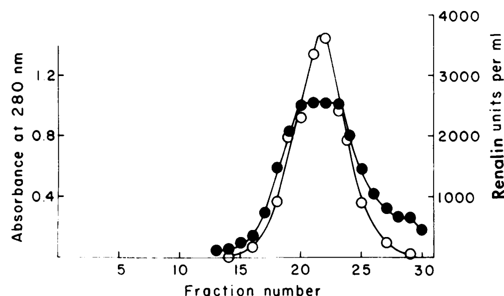

FIG. 1. Distribution of hemolytic (renalin) activity (○) and absorbance at 280 nm (●) among fractions eluted from a Sephacryl S-200 column.

The partially purified material had 7 to 13% of the activity of the original cultures and a specific activity of 4,500 to 9,000 RU per unit of absorbance at 280 nm (10-mm path). When the material was subjected to electrophoresis in polyacrylamide gels according to the method of Weber and Osborn (8), but in the absence of sodium dodecyl sulfate, staining revealed a major band together with some diffusely stained material which in some runs resolved into one to three bands. When an unstained gel was placed on a 10 mM $MgCl_2$–sheep erythrocyte agar plate beside a strip of filter paper wet with staphylococcal sphingomyelinase C, a well defined zone of hemolysis whose center coincided with the locus of the major stainable band developed at 37°C (Fig. 2). The major band, therefore, appeared to be renalin. Densitometric scanning of the gels suggested that the preparation was on the order of 50% pure.

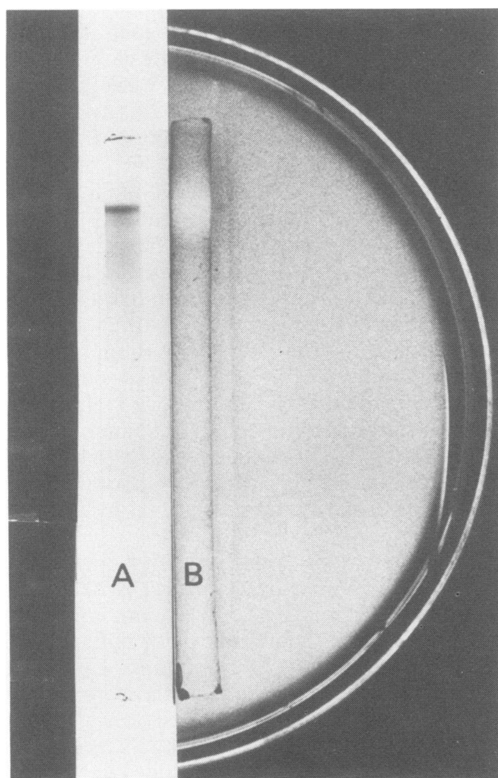

FIG. 2. (A) Band obtained by the staining of a polyacrylamide gel after electrophoresis of partially purified renalin. (B) Zone of hemolysis (white) produced by diffusion from an unstained gel that was juxtaposed with staphylococcal sphingomyelinase C on sheep erythrocyte agar.

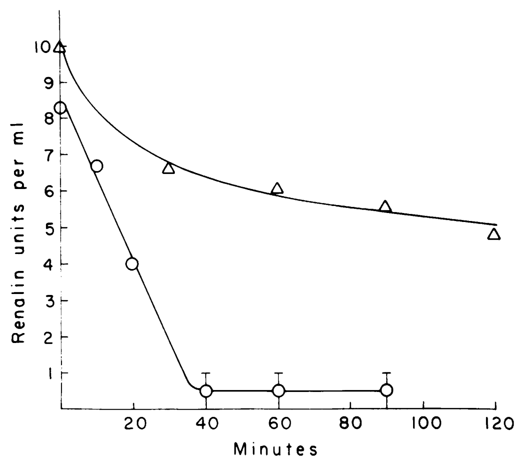

FIG. 3. Thermal inactivation of renalin at 60°C (△) and at 100°C (○).

An analysis of partially purified renalin by gel electrophoresis in the presence of sodium dodecyl sulfate revealed a major band and several fainter ones. By a comparison of the migration distance of the major band with those of standard proteins, the molecular weight of the former was estimated to be 63,000. A comparison of the elution volume of renalin activity in a Sephacryl S-200 column (Fig. 1) with those of standard proteins indicated a molecular weight of 58,000. An analysis by electrofocusing in a linear density gradient in a 110-ml column with conditions as described previously (2) revealed a single peak of renalin activity congruent with an absorbance peak (measured at 280 nm) at pH 4.6. The activity peak was symmetrical except for a low shoulder on the left limb.

Portions of a solution of renalin diluted to contain 10 RU/ml were placed in water baths at 60 and 100°C, and samples were assayed after being chilled at various time intervals. The active protein had a half-life of about 120 min at 60°C and 16 min at 100°C (Fig. 3).

When washed sheep erythrocytes were treated with a minute amount of staphylococcal sphingomyelinase C, washed, and then exposed

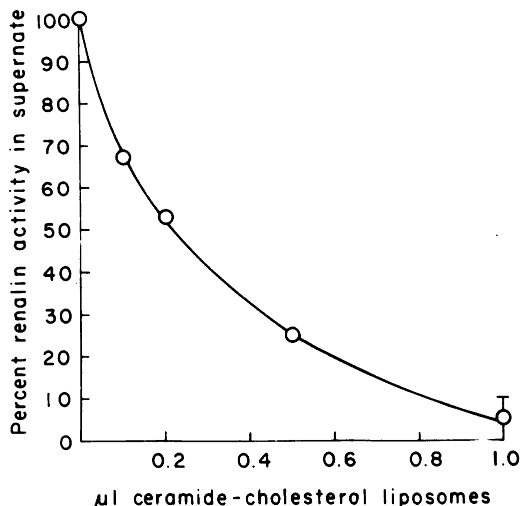

FIG. 4. Binding of renalin by ceramide-cholesterol liposomes.

to renalin, lysis occurred. When washed sheep erythrocytes were treated with renalin, washed, and then exposed to staphylococcal sphingomyelinase C, no lysis occurred. These results suggest that renalin only affects erythrocytes whose membranes have first been modified by exposure to the staphylococcal enzyme.

The products that result from the action of staphylococcal sphingomyelinase C on sheep erythrocyte membranes are ceramide (*N*-acylsphingosine) and phosphorylcholine (5). We postulated that renalin functions by the further modification of membranes that are already altered by the action of the staphylococcal enzyme. If this is true, it should be possible to demonstrate an interaction between renalin and ceramide. Liposomes containing ceramide and cholesterol were prepared as described previously (2). It was found that a very small amount of liposome suspension (0.08 µl) was sufficient to inhibit 1 U of renalin when the test amount of renalin was 2 U. Under the same conditions, the volume of liposomes containing sphingomyelin (in place of, but equimolar to ceramide) and cholesterol that was required to inhibit renalin was 15 µl, or 62 times the volume required with ceramide. Liposomes containing phosphatidylcholine (in place of, but equimolar to ceramide) and cholesterol did not inhibit renalin in concentrations up to 1,000 times as great as that of ceramide-containing liposomes. A suspension of sphingosine, equivalent in concentration to that of ceramide, inhibited renalin at a concentration 180 times that of ceramide.

These results showed that renalin reacts with ceramide with a considerable degree of specificity. The question then arose as to whether or not the action of renalin on ceramide was enzymatic. Samples of washed sheep erythrocytes were treated with (i) renalin and sphingomyelinase C and (ii) sphingomyelinase C only. After being chilled, the membranes were extracted by the method of Bligh and Dyer (3), and the extracted lipids were analyzed by thin-layer chromatography with Silica Gel G and chloroform-glacial acetic acid (96:4, vol/vol) for development (7). Spots were made visible by exposure to iodine vapor. Spots having the same migration distance as that of authentic ceramide were seen with both extracts. There was no diminution in staining intensity when renalin was used, nor were any new spots detected.

The results indicated that renalin interacts with ceramide nonenzymatically. In support of this, it could readily be demonstrated that small quantities of liposomes containing ceramide and cholesterol were able to bind renalin. Twofold dilutions of liposomes were allowed to interact with 10 RU in buffer 1. After 30 min at 30°C, the liposomes were sedimented by centrifugation at 17,000 × *g* for 60 min. The supernatants were then assayed for renalin to determine what portion had been bound. A total of 0.2 µl of liposomes bound half the renalin in the system, and larger amounts of liposomes bound nearly all the renalin (Fig. 4). A curve closely similar to that of Fig. 4 was obtained when the experiment was done at about 0 instead of 30°C.

We conclude that *C. renale* produces an extracellular protein, renalin, that has a strong affinity for ceramide. Under the conditions used, renalin appears to react nonenzymatically with ceramide and causes lysis by combining with membrane ceramide generated by the previous action of staphylococcal sphingomyelinase C. The action of renalin seems to resemble that of the CAMP protein of *S. agalactiae*, but renalin (molecular weight of 60,000; pI of 4.6) differs physically from the CAMP protein (molecular weight of 23,500; pI of 8.3).

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LITERATURE CITED

1. **Bernheimer, A. W., L. S. Avigad, and K. S. Kim.** 1974. Staphylococcal sphingomyelinase (β-hemolysin). Ann. N.Y. Acad. Sci. **236**:292–306.
2. **Bernheimer, A. W., R. Linder, and L. S. Avigad.** 1979. Nature and mechanism of action of the CAMP protein of group B streptococci. Infect. Immun. **23**:838–844.
3. **Bligh, E. G., and W. J. Dyer.** 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37**:911–917.
4. **Christie, R., N. E. Atkins, and E. Munch-Petersen.** 1944. A

note on a lytic phenomenon shown by group B streptococci. Aust. J. Exp. Biol. Med. Sci. 22:197–200.

5. **Doery, H. M., B. J. Magnusson, J. Gulasekharam, and J. E. Pearson.** 1965. The properties of phospholipase enzymes in staphyloccal toxins. J. Gen. Microbiol. **40**:283–296.
6. **Fraser, G.** 1964. The effect on animal erythrocytes of combinations of diffusible substances produced by bacteria. J. Pathol. Bacteriol. **88**:43–53.
7. **Randerath, K.** 1968. Thin layer chromatography, 2nd ed., p. 172. Academic Press, Inc., New York.
8. **Weber, K., and M. Osborn.** 1969. The reliability of molecular weight determination by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. **244**:4406–4412.