Simple Procedure for Production by Group C Streptococci of Phage-Associated Lysin Active Against Group A Streptococci

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Phage-associated lysin of high potency was prepared by growing the host group C streptococcal strain 26RP66 in a semisynthetic medium. The lysin was stabilized by adding dithiothreitol and neutralized ethylenediaminetetraacetic acid (EDTA) to facilitate further concentration and partial purification. The lysin remained active when stored at −65 C for 1 year. Lysin was active against all strains of group A streptococci tested and was more active against living cells than heat-killed cells. The procedure outlined is practicable for most bacteriological research laboratories and does not require column purification or other complex biochemical procedures. It should be useful to any laboratory which requires small amounts of lysin to produce L-forms and protoplasts or to release streptococcal antigens.

Phage-associated lysin is a lytic enzyme which appears in phage lysates coincident with lysis of the host bacterium. Phage-associated lysin, active against streptococci of groups A, C, and E, was first observed by Evans in 1934 as a phenomenon which she called "nascent lysis" (5). Maxted in 1957 showed that this lytic activity was due to a phage-associated lysin which was released when the group C streptococcal strain 26RP66 was infected by a virulent phage called C' (14). The lysin was inactivated by oxidation and it lysed living streptococci more readily than heat-killed streptococci. Similar results were reported by Krause (11, 12).

Phage-associated lysin has been used to release streptococcal antigens, such as M protein (9, 10, 12), T protein (4), and C carbohydrate (12). L-forms (8) and protoplasts (7) have been prepared from group A streptococci by action of phage-associated lysin in hypertonic solutions. Difficulties in preparation and stabilization of this lytic enzyme, however, have limited its use in streptococcal research. Recently Fischetti et al. (6) reported a method by which ample amounts of purified lysin could be prepared. Their procedure required the stabilization of the SH groups of the lysin by reaction with tetra-thionate. The stabilized, but at this point inactive, lysin was then subjected to ammonium sulfate precipitation and column purification. After separation and purification, the lysin was reactivated by adding Cleland's reagent dithiothreitol (DTT). This complex procedure would be adequate only if a central laboratory were able to make enzyme available to smaller laboratories which lack the biochemical capability required.

For several years we have produced phage-associated lysin from the group C strain and propagated the phage in a semisynthetic medium. This medium contains no peptones and proteases other than those carried over with the inoculum. The use of this simple medium facilitated the separation and partial purification of the lysin. DTT and freezing at −65 C stabilized the phage-associated lysin for future use as a lytic agent in streptococcal research.

MATERIALS AND METHODS

Cultures. The group C Streptococcus 26RP66 and phage C' were obtained from Roger M. Cole of the National Institutes of Health, Bethesda, Md. The group A, type 1, Streptococcus pyogenes strain DS 2036-66 was obtained from Joseph Padula of the Center for Disease Control, Atlanta, Ga.

Medium. The semisynthetic medium used to grow strain 26RP66 for lysin production and phage propagation has been previously reported (2). The following changes were made. The medium was buffered at pH 7 with 0.06 M potassium phosphate, and addition of 5 g of potassium phosphates, as listed in the original formula (2), was omitted.

Inocula. Stock cultures of strains 26RP66 and DS 2036-66 were grown in Todd-Hewitt broths (Difco), washed once in 0.05% sterile neutralized cysteine-hydrochloride, and frozen in small portions at −65 C. Phage C' was also kept frozen at −65 C for long-term storage. Phage C' for propagation and lysin production was often kept at 4 C for several months without significant loss in titer.

Phage titration. Phage C' was estimated by
plaque counts on Todd-Hewitt medium containing 1.5% Noble agar (Difco). Plates were flooded with streptococci from an overnight Todd-Hewitt or Trypticase soy (BBL) broth cultures of 26RP66, and the excess was aspirated and removed by capillary pipette. The plates were then incubated at 37 C for 2 h to allow the streptococcal layer to dry. Then appropriate dilutions of phage C' were dropped on the plates with a tuberculin syringe equipped with a 27-gauge needle. Phage dilutions were usually made in broth, although distilled water or saline also were suitable.

Lysozyme production and phage propagation. Flasks of semisynthetic medium (300 ml in each 500-ml Erlenmeyer flask) were inoculated with 5% (vol/vol) of a 16-h Trypticase soy broth culture of 26RP66. The flasks were incubated at 37 C in a water bath for 3 to 4 h until the turbidity registered an absorbance of 0.03 ± 0.03 at 550 nm in a Coleman junior spectrophotometer. Phage C' was added at a multiplicity of infection of about 2 to 6. The phage-cell suspension was mixed thoroughly and then centrifuged at 39,000 relative centrifugal force for 1.5 h at 4 C. This procedure, suggested by Barkulis et al. (1), clarified the lysozyme solution and facilitated the precipitation of lysozyme by ammonium sulfate. The precipitate, formed when streptomycin sulfate was added, also contained entrapped phage C' which could be recovered in high concentration. Phage C' was recovered by suspending the pellet in about 3 ml of saline, and the streptomycin sulfate was removed by dialysis against 0.02 M, pH 7.4, phosphate buffer. Phage C' titered 10<sup>12</sup> to 10<sup>13</sup> plaque-forming units/ml were obtained. These phage concentrations were at least 100-fold higher than the best liquid or semisolid propagations that we have achieved without an additional concentration step.

Lysozyme was precipitated by adding solid ammonium sulfate at 4 C with constant stirring to a final concentration of 50% saturation. The precipitation was allowed to proceed at 4 C for 24 to 72 h, and the lysozyme was separated from the fluid by centrifugation at 39,000 relative centrifugal force for 1.5 h at 4 C. The lysozyme was recovered from the walls of the beaker in which it precipitated and from the precipitate in a small amount of 0.02 M phosphate buffer (pH 6.1) containing, in a final concentration, 0.001 M DTT and 0.006 M ethylenediaminetetraacetic acid. About 6 ml of buffer was used to take up the lysozyme from 400 ml of crude C' lysate. The concentrated lysozyme was centrifuged at 12,000 relative centrifugal force for 45 min at 4 C. The pellet, containing insoluble material including a small amount of phage C', was discarded, and the clear supernatant fluid with its active lysozyme was frozen at −65 C.

**RESULTS AND DISCUSSION**

In Table 1 the results of an experiment in which lysin was added to 7-h-old cultures, 30-h-old cultures, and cultures heat-killed at 56 C for 1 h are shown. Cultures grown for 30 h were slightly less sensitive to lysin than cultures harvested at 7 h, but heat-killed cultures were much less sensitive to lysin than the living cultures. Mixed (14) reported similar results when comparing the effects of lysin on living streptococci, heat-killed streptococci, and chloroform-killed streptococci. These results suggest that autolysins present in living streptococci potentiated the lytic action of phage-associated lysin. In our experiments, significant lysis of living streptococci was observed at a 1:800 dilution of lysin (Table 1).

The effect of storage at −65 C on the activity of phage-associated lysin (protected from inactivation by DTT and ethylenediaminetetraacetic acid) is shown in Table 2. After a year of storage, a measurable drop in lytic activity was observed, but the residual activity was high and the preparation was useful for experiments involving the lysis of group A streptococci.

Our primary interest in phage-associated lysin has been as a tool for the release of streptococcal antigens at 37 C and near neutral pH. Therefore, another group A streptococcal

| Lysin dilution | Transmission at 550 nm (%) |
|----------------|---------------------------|
|                | 7 h living<sup>a</sup> | 30 h living<sup>a</sup> | Heat-killed<sup>a</sup> | 7 h<sup>b</sup> |
| 1:50           | ND<sup>d</sup>          | 90                      | 80                     | 91            |
| 1:100          | 99                      | 89                      | 77                     | 85            |
| 1:200          | 98                      | 86                      | 74                     | 83            |
| 1:400          | 94                      | 85                      | 68                     | 78            |
| 1:800          | 83                      | 81                      | 69                     | ND            |

<sup>a</sup> Cultures were incubated with lysin for 10 min at 37 C; reaction was stopped by plunging tubes into a boiling water bath.

<sup>b</sup> Cultures were grown for 16 h at 37 C prior to heat-killing at 56 C for 1 h.

<sup>d</sup> Cultures were incubated at 37 C for 10 min and then held in an ice bath until read in the spectrophotometer.

<sup>Not done.</sup>
strain, 0098 (type 13), was tested under conditions similar to those by which we assayed lysin with DS 2036-66. The results (Table 1, right column) show substantial lysis of type 13 at a lysin dilution of 1:400. In qualitative tests, other group A streptococci, including another M type 1 strain (0001/B1) and M-negative T type 1 strain (DS 1134-66) and types 5, 6, 12, 23, and 24, were readily lysed by phage-associated lysin. These results agree with prior reports of the general susceptibility of group A streptococci to the phage-associated lysin released when phase C' infects a susceptible group C strain, such as 26RP66 (1, 3, 6, 11, 14).

Since we have been concerned with the problem of estimating the amount of lytic activity in various lots of lysin, we were also concerned about the effect of time on lysis. The results (Table 3) show the effect of incubation time on lysis with various concentrations of lysin. In this experiment, tubes containing 7-h cultures of DS 2036-66 and appropriate dilutions of the lysin were incubated at 37 C and later removed at various intervals to an ice bath. After 4 min, little lysis had taken place even with the low dilutions of lysin. After 40 min, however, a 1:800 dilution of lysin resulted in substantial lysis of the test culture. These kinetics are entirely opposite from those observed with an enzyme such as trypsin. Trypsin and other proteolytic enzymes attack a substrate, such as casein, in such a way that in the initial stages of the reaction the hydrolysis and release of soluble peptides takes place at a maximal rate (13). In contrast, in the case of a lysin, the lytic reaction, by which cells are disrupted and their contents solubilized, is a secondary effect caused by the hydrolysis or breakage of important groups in the polymers of the cell wall.

Other investigators have reported that phage-
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