IMMUNOLOGICAL PROPERTIES OF HYALURONIDASES ASSOCIATED WITH TEMPERATE BACTERIOPHAGES OF GROUP A STREPTOCOCCI*

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The presence of hyaluronidase associated with temperate bacteriophages from group A streptococci has been well established (1–3). The enzyme appears in lysates of strains of group A streptococci infected with temperate phages (1, 3). All of the hyaluronidase activity present in such phage lysates is not associated with sedimentable phage. A major portion of this enzyme activity is present in phage-free lysis supernates as free enzyme or on phage fragments. The remaining enzyme, about one-fourth to one-third of the total, is tightly bound to the viral particle and may be an integral element of the bacteriophage (1, 3). In this communication, the two sorts of phage-associated hyaluronidase will be referred to as the free and the bound enzyme.

Earlier studies have suggested that at least four serological types of phage-associated hyaluronidase exist (4). These appear to be immunologically unrelated to streptococcal hyaluronidase, the extracellular enzyme produced by group A streptococci in the absence of phage infection. The serological specificity of the phage-associated enzymes seems to parallel that of the M-type of the host strain. Antisera from rabbits immunized with crude phage lysates neutralize the homologous phage-associated hyaluronidase and also inhibit propagation of the homologous phage, suggesting the possibility that these effects may be due to the same antibody (4).

These previous studies have been carried out with crude lysates of considerable complexity in composition. In recent studies from this laboratory the hyaluronidase associated with a temperate bacteriophage of a strain of M type 49 group A streptococci has been purified and characterized (5). The availability of this homogeneous protein has allowed a more precise examination of the immunological interrelationships of phage and bacterial enzymes which comprises the present report.

Materials and Methods

Streptococcal Strains and Bacteriophages. The temperate bacteriophages used in this study were obtained from strains of group A streptococci by induction with mitomycin C as previously described (6). The strains were from the collection of this laboratory and have been described elsewhere (7, 8). Unless stated otherwise, the M type 12 group A strain designated as K56 and originally received from Kjems (1) was used as a standard indicator to prepare lawns. Mitomycin C-induced phage lysates were prepared as previously described (6). Lysates were

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cleared of cellular debris by low speed centrifugation. Thereafter the supernates were passed through membrane filters (HA type, 0.45 μm pore size; Millipore Corp., Bedford, Mass.). Bacteriophage titers of the lysates and K values of antisera were determined as reported earlier (6, 8). The method for phage subtyping of the M type 49 group A streptococci that were used in this study has been described elsewhere (6).

Media. Broth for bacterial growth and induction of phages from stock strains and agar plates for plaque assays of the bacteriophages have been described elsewhere (6). Hyaluronidase (bovine testes; Sigma Chemical Co., St. Louis, Mo.) was added as indicated at a concentration of 40 μg/ml in plates.

Hyaluronidase Assay. Hyaluronidase activity was determined by following the extent of degradation of hyaluronic acid (human umbilical cord; Sigma Chemical Co.) by measuring the remaining substrate by a sensitive spectrophotometric method. This assay of enzyme activity is based on the binding of a carbocyanine dye to undegraded substrate and has been previously described (9). The enzyme unit has been defined as that amount of hyaluronidase resulting in a 10% decrease in the absorbance of the dye-hyaluronic acid complex after incubation of the polysaccharide for 1 h at 37°C and at pH 5.0, half of the activity is obtained at pH 7.4 (5).

Streptococcal Phage-Associated Hyaluronidases. Phage-associated hyaluronidase activities, bound to temperate bacteriophage from a group A type 49 streptococcus (University of Minnesota strain GT-8760) or present in phage-free supernate from lysates of the same phage strain have been described in earlier reports (3, 9). For the present studies, a purified form of the bound enzyme was prepared as previously detailed (5). The enzyme was released by urea treatment and purified to homogeneity as determined by sodium dodecyl sulfate-polyacrylamide electrophoresis.

Bacterial (Extracellular Streptococcal) Hyaluronidase. A crude enzyme preparation was obtained by concentrating 1.5 liters of the culture supernate of an M type 4 group A streptococcus (University of Minnesota strain 77-295) at 4°C with ammonium sulfate to 85% saturation.

Bovine Testicular Hyaluronidase. A partially purified preparation of this enzyme was obtained from Sigma Chemical Co.

Immunological Procedures. Immune sera against preparations of purified hyaluronidase and whole temperate bacteriophage (obtained from strain GT-8760) purified as previously described (5) were produced in New Zealand white rabbits. Each animal was immunized every day with about 5×10^{10} plaque-forming units (PFU)[1] or 150 μg of purified enzyme for a total of 20 intravenous injections. The animals were bled 5 days after the administration of the last injection and antisera were tested for the ability to neutralize bacteriophages. The experiments of phage neutralization were carried out according to the method of Adams (10) with the use of freshly prepared mitomycin C-induced lysates. Precipitin tests were performed in 0.8% agarose and 10 mM sodium phosphate buffer (pH 7.0) according to the double-diffusion method of Ouchterlony (11). Immunoprecipitates were obtained as described by Weeke (12).

For testing hyaluronidase inhibition, preparations of immunoglobulins were used to reduce the background obtained when whole antisera were used. Antiserum to the temperate bacteriophage or to hyaluronidase was applied onto a column (1.5 x 28 cm) of Sephadex G-100 and eluted with 10 mM tris (hydroxymethyl) amino-methane (Tris)-hydrochloride buffer at pH 7.4. Immunoglobulins were recovered (along with other high molecular weight proteins) from the fractions eluted in the void volume of the column by precipitation with 85% saturated ammonium sulfate, dialyzed for 18 h at 4°C against Tris-hydrochloride buffer and reconstituted to their original volume. Protein concentrations in these immunoglobulin preparations were determined by using the method of Bradford (13) with crystalline bovine serum albumin as standard.

The hyaluronidase-inhibiting power of these immunoglobulin preparations was tested in a system of three components (enzyme, antibody, and substrate) as follows: immunoglobulins were added in increasing amounts (5–30 μg) to 3.0 U of enzyme contained in (*a*) bacteriophages that had been purified by equilibrium centrifugation in cesium chloride gradients (3), (*b*) the supernate of a phage-free lysate or (*c*) purified bound hyaluronidase. The reaction mixtures in an 0.08 ml vol containing 0.8 μmol of Tris-hydrochloride at pH 7.4 were incubated in microcentrifuge tubes, capacity 0.4 ml (Stockwell Scientific, Monterey Park, Calif.), for 30 min

[1] *Abbreviation used in this paper*: PFU, plaque-forming unit.

at 37°C and then centrifuged at 500 *g* for 20 min to separate immunoprecipitates and supernates. 5 μg Hyaluronic acid substrate was added to 0.045-ml samples of the supernatant fractions in a final 0.05 ml vol, and incubations were carried out at 37°C for 18 h. One drop of toluene was added to prevent bacterial growth. 0.27 ml water and dye were subsequently added to 0.03-ml samples of the incubation mixtures to a final 3.0 ml vol and absorbance measured at 640 nm (9). The controls contained (*a*) hyaluronic acid and immunoglobulins of rabbits immunized with whole temperate phages or with purified bound hyaluronidase, (*b*) hyaluronic acid, bovine testicular hyaluronidase and the same immunoglobulins, (*c*) hyaluronic acid and either temperate bacteriophages or purified bound hyaluronidase, (*d*) hyaluronic acid and either broth or serum from nonimmunized animals. Assays were performed in triplicate. The effect of various immunoglobulin preparations on enzyme function was expressed as the percentage of inhibition of initial enzymatic activity.

The effects of immunoglobulins from antisera for purified bound enzyme and for whole temperate phage (from strain GT-8760) on the enzymatic activities present in mitomycin C-induced lysates were also examined as follows: phage lysates (0.04 ml; after clearing from cellular debris by low speed centrifugation and dialysis against 10 mM Tris buffer, pH 7.4) were incubated with immunoglobulin preparations in a final 0.08 ml vol at 37°C for 30 min (which is long enough to give maximal inhibition) after which the tubes were centrifuged and the hyaluronidase activity determined in the supernate after addition of hyaluronic acid to the system as described above. The above mentioned controls were also included.

Results

Specificity of Antibody to the Purified Bound Enzyme as Determined by Double Diffusion in Agar. The ability of antibody against a purified preparation of bound hyaluronidase (from a temperate bacteriophage of an M type 49 group A streptococcus) to produce precipitin reactions with hyaluronidase preparations from different sources was examined by Ouchterlony double diffusion in agar (Figs. 1 and 2).

As shown in Fig. 1, antiserum to the purified bound enzyme (well 6) reacted with the homologous free enzyme (well 5) as well as with the homologous bound enzyme (center well), forming a single line of identity. Similar reactions were obtained with an immunoglobulin fraction of this antiserum (well 3) against the homologous free (well 2) and bound (center well) enzymes. No reaction was observed between the antiserum to the purified bound enzyme (well 6) and bovine testicular hyaluronidase (well 1). In addition, no reaction was seen between the immunoglobulin fraction from antiserum to the purified bound enzyme (well 3) and the free enzyme from an induced lysate of a strain of a heterologous type (well 4).

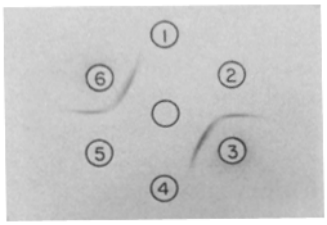

FIG. 1. Ouchterlony plate showing the specificity of the antiserum and immunoglobulin fraction obtained from rabbits immunized with the purified bound hyaluronidase. Center well: purified bound enzyme from temperate phage of type 49 streptococcus (strain GT-8760). Well 1: bovine testicular hyaluronidase. Wells 2 and 5: homologous free enzyme (phage-free supernate from mitomycin C-induced lysate of strain GT-8760). Well 3: immunoglobulin fraction from antiserum to homologous purified bound enzyme. Well 4: heterologous free enzyme (phage-free supernate from mitomycin C-induced lysate of a type 12 streptoccus, strain GT-2172). Well 6: whole antiserum to homologous purified bound enzyme.

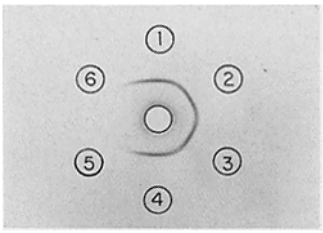

FIG. 2. Ouchterlony plate showing the immunological identity of free enzyme from phage-free mitomycin C-induced lysates of four different strains of type 49 streptococci. Center well: immunoglobulin fraction from antiserum to purified bound enzyme from temperate phage of type 49 streptococcus (strain GT-8760). Wells 1–4: free enzyme (concentrated phage-free supernates) from mitomycin C-induced lysates of type 49 strains GT-8760, GT-7907, GT-9278, and GT-7903, in order (See Table I for details on strains). Wells 5–6: free enzyme (similarly obtained) from lysates of type 12 strains GT-2172 and GT-9211S.

Fig. 2 shows the reactions of the immunoglobulin fractions of antiserum against the purified bound enzyme (center well) with preparations of free enzyme from strains of the same and of a different M type. A single precipitin line of identity was found with free enzyme preparations (phage-free supernates of induced lysates) from four strains of homologous (type 49) M type. These M type 49 strains were of different phage subtypes as determined by lytic patterns (6) and from different epidemiological incidents, i.e. diverse geographical or temporal sources (Table I). No reaction was obtained with similar preparations of free enzyme from induced lysates of two strains of a heterologous M type (type 12), strains GT-2172 and GT-9211S, from Cleveland and from Copenhagen, respectively (8).

No cross-reaction with other streptococcal antigens was observed with antiserum against purified bound enzyme in experiments (not shown) using ammonium sulfate-precipitated extracellular streptococcal products (including bacterial hyaluronidase) from the supernate of an M type 4 group A strain (University of Minnesota strain 77-295). In addition the purified bound enzyme did not react with human antiserum for serum opacity factor or with rabbit antisera for either M or T proteins of type 49 streptococci.

Neutralization of Phage-Associated (Bound and Free) Hyaluronidase by Antibodies to Purified Bound Enzyme and to Whole Phage. The effects of the antibodies against purified bound hyaluronidase and of antibodies against homologous whole phage upon the activities of the homologous bound and free enzymes are illustrated in Fig. 3. The immunoglobulin fractions obtained by gel filtration of the antisera against purified bound hyaluronidase or the homologous whole phage were used in these enzyme neutralization experiments (Materials and Methods). When increasing amounts of immunoglobulin against the purified bound enzyme were added to a constant amount (1.0 U) of this enzyme, a progressive decrease was obtained in the enzymatic activity. The neutralization curve (●) indicates that the inhibition reached more than 95% of the initial activity and was maximal with 5 μg of immunoglobulins. The residual activity could not be neutralized even after the addition of 50 μg of immunoglobulins per unit of enzyme (not shown). It is likely that the inhibition of hyaluronidase is due to interaction with the antibodies since a preparation of heat-denatured immunoglobulins (heated for 10 min in boiling water) had no effect on enzyme activity at concentrations ranging from 5 to 15 μg per unit of enzyme. A similar pattern of neutralization (▲) was observed when this immunoglobulin preparation was examined

TABLE I

Inhibition of Enzyme Activities Present in Induced Lysates of Various Strains of Group A Streptococci by Antibody against Purified Bound Hyaluronidase from Temperate Phage from a Type 49 Strain (GT-8760)

Induced lysate* of strain	Serological classification of strain		Inhibition of hyaluronic acid degradation‡
	M	T	
			%
GT-2914 (C203S)	3	1	12
75-194	4	4	6
70-086	5	5/25/44	5
GT-9440§	6	6	9
GT-2185	11	11	17
GT-9277 ‖	12	8/12	8
70-089 ‖	12	12	6
GT-9211S	12	12	1
GT-9683	12	12	13
GT-6898 ‖	12	12	7
GT-7817 ‖	12	12/B3264	11
GT-6527	14/51	14	4
GT-6525	24	NT‡‡	3
70-075	27	5/27/44	15
CV-162	41	3/13/B3264	12
GT-2184 ‖	44	12	13
GT-7907	49	49	88
GT-9278¶	49	49	93
GT-7903	49	49	85
GT-6365	41/52	3/13/B3264	10
CV-832	53	3/13/B3264	18
CV-581	54	15/17/19/23/47	9
70-185	55	8/25	14
70-184	57	8/25	11
GT-8237§	Neg.**	11/12	7

* Obtained after induction of phages from stock strains by mitomycin C.

‡ Immunoglobulins (25 μg) obtained after gel filtration of the antiserum for purified hyaluronidase were added to 0.04-ml samples of whole lysates (6 × 10^6 PFU/ml). Residual enzyme activity was measured on the supernatant fractions after centrifugation and addition of hyaluronic acid as described under Materials and Methods.

§ Assayed on lawns of a type 12 strain (strain 8747).

‖ Assayed on lawns of strain 9211S.

¶ Assayed on lawns of strain 7907.

** M-negative, classified as type 59 by serum opacity reaction.

‡‡ NT—Not typable by T agglutination.

for inhibitory activity on the homologous free hyaluronidase. The inhibition reached more than 90% of the initial activity. An immunoglobulin preparation from an antiserum for homologous purified whole temperate bacteriophage was also strongly inhibitory for the purified bound enzyme (△) and for the free enzyme (○) with similar levels of residual activities (5%) obtained at a ratio of 10 μg of immunoglobulin fraction protein to 1.0 U of enzyme.

The preceding experiments were performed with soluble preparations of the bound and free enzyme. Enzyme neutralization experiments were also performed with

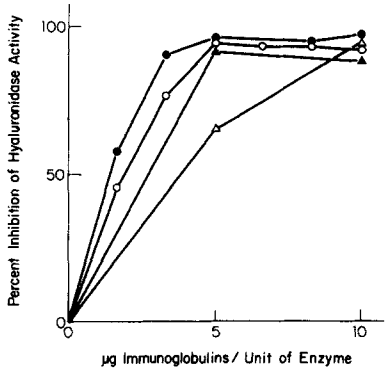

FIG. 3. Inhibition of homologous bound and free hyaluronidase activity by antibody to the purified bound enzyme and by antibody to the whole phage. Purified bound enzymes (1.0 U) obtained from the temperate phage from type 49 streptococcus (strain GT-8760) was incubated with immunoglobulin preparations obtained after gel filtration of antisera against homologous purified bound enzyme (●) or against homologous purified whole phage (△). A phage-free supernate of a mitomycin C-induced lysate of strain GT-8760 containing homologous free enzyme (1.0 U) was incubated with immunoglobulins from antisera against homologous purified bound enzyme (▲) or against homologous purified whole phage (○). After centrifugation to remove immunoprecipitates, residual (unneutralized) enzyme activity was measured on the supernates and the percent inhibition of enzyme activity was calculated (Materials and Methods).

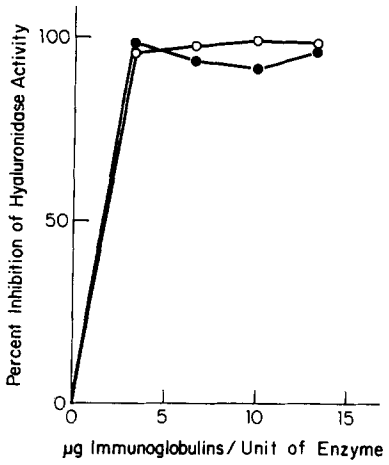

FIG. 4. Inhibition of the bound enzyme in its natural state (still bound to phage) by antibody to the homologous purified bound enzyme and by antibody to homologous purified whole phage. Purified whole temperate phage from a type 49 streptococcus (strain GT-8760), representing 1.0 U of enzyme, was incubated with an immunoglobulin preparation obtained after gel filtration of antisera against homologous purified bound hyaluronidase (○) or against homologous purified whole phages (●).

purified whole phage (Fig. 4). These experiments indicate that the bound enzyme in its natural state is also strongly inhibited by immunoglobulin fractions from rabbits immunized with a purified preparation of homologous bound enzyme or with homologous whole phage purified by equilibrium centrifugation in cesium chloride gradients. The inhibition reached nearly 100% with the antibody against the purified bound hyaluronidase. In additional experiments (not shown), the temperate bacteriophages from three other type 49 strains (strains GT-7907, GT-9278, and GT-7903)

TABLE II

Neutralization of Phage Propagation by Antibody to Purified Bound Hyaluronidase

Strain source of induced bacteriophage lysates*				K-value of antiserum§
Strain‡	M type	Phage subtype	Epidemiological incident	
GT-8760	49	I	Red Lake, 1966	32
GT-7907	49	II	Netherlands, 1962	29
GT-9278	49	III	Alabama, 1967	36
GT-7903	49	IV	Red Lake, 1953	25
GT-2172	12	—	Cleveland, 1953	5

* Lysates were prepared by induction with mitomycin C.

‡ Further details on strains are provided in previous publications (6, 8).

§ The immunoglobulin preparation against purified bound hyaluronidase from temperate phage of a type 49 streptococcus (strain GT-8760) was diluted at 1:250 and added to filtered phage lysates. The mixture was incubated at 37°C for 30 min before plating on an indicator strain. The K-value was calculated from the reduction in PFU (Materials and Methods).

were similarly purified. Treatment of the phages with 15 μg of the immunoglobulin fraction from antiserum to the purified bound enzyme resulted in a loss of hyaluronidase activity of about 90% (a loss similar to that depicted in Fig. 4). This suggests that these phage-bound enzymes from diverse strains of type 49 streptococci (Table I) share antigenic determinants.

When bovine testicular hyaluronidase or a preparation of extracellular hyaluronidase from an M type 4 group A streptococcus (strain 77-295) was treated with 15 μg of the immunoglobulin fraction from antisera against the purified bound enzyme only minimal inhibition (2–10%) of the catalytic activity of the enzymes was observed (not shown).

Absence of Neutralization of Enzyme Activity in Induced Lysates of Streptococcal Strains of Heterologous M Types by Antibody to Purified Bound Enzyme. The relationship between phage hyaluronidase activity in induced lysates of streptococcal strains of various M types was examined using the immunoglobulin preparation from rabbits immunized with the purified bound enzyme from the phage of an M type 49 strain (GT-8760). Since the phage-bound and free hyaluronidases in lysates of M type 49 strains are immunologically identical, the lysates from the various strains were compared without prior removal of the phage particles. The extent of inhibition of hyaluronidase activity is shown in Table I. Although minimal inhibition (18% or less) was observed in several lysates from strains of heterologous M types, only those from M type 49 strains were markedly affected (85% or greater) by the antibody preparation. The hyaluronidase activity associated with bacteriophages from M type 49 streptococci is clearly not closely related immunologically to the enzymes from most of the other bacteriophages, as indicated by these studies of inhibition of enzyme activity.

Inhibition of Propagation of Temperate Phages from Diverse Strains of the Same M Type by Antibody to Homologous Bound Enzyme. The immunoglobulin fractions of the antiserum against purified bound enzyme derived from temperate bacteriophage of an M type 49 streptococcus (strain GT-8760) was tested for its ability to inhibit the propagation of bacteriophages from induced lysates of four strains of type 49 streptococci, representative of different phage subtypes and from different outbreaks. The results of this study (Table II) also indicate the relatedness of the phage enzymes from diverse

strains of M type 49 streptococci because the propagation of all of these phages was inhibited by this immunoglobulin preparation. In contrast the propagation of a phage from an induced lysate of an M type 12 strain was relatively unaffected by this antibody preparation. Identical K values were calculated for the anti-enzyme with or without added bovine testicular hyaluronidase in the plates.

Immunological Relationship of Extracellular Streptococcal Hyaluronidase to Phage-Associated Hyaluronidase. Streptococcal extracellular hyaluronidase is abundantly produced by M type 4 strains and one of these (strain 75-194) was used to compare the bacterial enzyme and the phage-associated enzyme, both derived from the same strain. The extracellular hyaluronidase from this organism was strongly inhibited (84%) by a human serum that had given an anti-streptococcal hyaluronidase titer of 512 by the conventional mucin clot prevention method (14). This serum had little effect on the free hyaluronidase activity present in a mitomycin-induced lysate of the same strain (10% inhibition). The uninduced culture supernate contained approximately 4×10^2 PFU/ml (due to spontaneous release from the lysogenized bacteria) while the induced lysate contained 6×10^6 PFU/ml although the uninduced culture supernate and the phage lysate contained roughly similar levels of hyaluronidase activity. Therefore phage hyaluronidase did not appear to represent a significant proportion of the enzyme activity of the uninduced culture supernate. The residual uninhibited activity in the induced lysate may be a function of the methodology or may indicate that some slight cross-reactivity exists between the phage and bacterial enzyme.

As the previous results (Table I) would predict, the anti-type 49 phage hyaluronidase immunoglobulin preparation had very little (6%) inhibitory effect on the enzyme activities of induced or noninduced type 4 culture.

Discussion

The more extensive and definitive results of the present study employing antibody to purified phage enzyme and antibody to purified phage are in accord with the earlier conclusions of Kjems (1, 4), from experiments in which crude phage lysates were used.

In the experiments reported here, immunodiffusion studies and a functional enzyme-inhibition assay using antibody to purified hyaluronidase associated with a temperate bacteriophage from an M type 49 group A streptococcus indicated that the phage-bound and free hyaluronidases present in mitomycin-induced lysates of this strain are serologically identical. Hyaluronidases of temperate bacteriophages from type 49 strains from different epidemiological incidents and of four phage subtypes were also antigenically indistinguishable. In addition, the similarity in the patterns of enzyme neutralization by antibody against whole temperate phage provided evidence that phage hyaluronidase is available as antigen in the intact viral particles. Antibody to the purified enzyme also inhibited propagation of temperate phages from strains of the same M type, supporting the view of Kjems that the enzyme-neutralizing and phage-inhibiting antibody may be the same.

The availability of antibody against a purified preparation of phage-associated hyaluronidase has allowed further and more precise examination of the relationship between enzymes of phages from strains of different M types. It is evident that immunological homology exists between the hyaluronidases of phages from the same M-type organisms. Phage-associated enzymes from strains of other M types are

inhibited to a relatively low extent by the M type 49 phage anti-hyaluronidase suggesting that the degree of homology is minor with enzymes associated with phages from heterologous M types. These studies depend on functional inhibition and may not measure antibodies directed against antigenic determinants unrelated to the active site of the enzyme. However, if immunological similarities exist between the enzymes, these do not result in immunoprecipitates detectable by the Ouchterlony technique.

The reason for this remarkable heterogeneity in phage-associated hyaluronidases is not clear. The enzymes apparently perform the same function of penetrating the host capsule and it would seem that they should have similar active sites. It is possible that the principal blocking antibodies are not directed against the active site but against some adjacent structure which varies in the different phages.

The hyaluronidase from streptococcal phage and that of bacterial origin may be similar in some properties (5). There is, however, little immunological resemblance between these enzymes. The human antiserum strongly inhibitory against a streptococcal extracellular hyaluronidase from an M type 4 strain had little inhibitory effect on the phage-associated enzyme in induced lysates of the same bacterial strain. The immunological heterogeneity observed in the phage-associated hyaluronidases of group A streptococci does not occur in the bacterial enzyme which appears to be group-specific rather than type-specific (15).

The association of immunological specificity of phage hyaluronidase with the M type of the strain of phage origin suggests that the phage enzyme might share an antigenic determinant with M protein or some other relatively type-specific antigen of the bacterium or that the production of specific enzyme might be influenced by the bacterium. With respect to the former, no reaction could be demonstrated by double diffusion in agar between purified phage enzyme and antisera to M protein, T protein or the serum opacity reaction factor prepared with a strain of the same antigenic composition. With respect to the second possibility, the strain almost universally employed as an indicator is K56, an M type 12 streptococcus. The growth of phage in this M type 12 strain does not influence the nature or specificity of the phage hyaluronidase. In the present study, phages were routinely propagated on this strain, yet, the results clearly demonstrate type specificity of the hyaluronidase. The curious parallelism between the immunological specificity of the phage and the M type of the strain of origin remains unexplained.

Summary

The antigenic relationships of hyaluronidases, bound and free, associated with temperate bacteriophages of group A streptococci were examined with antibody against purified whole phage and with antibody against phage-bound enzyme released by urea and purified to homogeneity.

Studies performed by double diffusion in agar (Ouchterlony) with antibody against the homologous purified enzyme from a temperate phage of a type 49 streptococcus indicated that the bound and free enzyme gave a single line of identity and that the free hyaluronidase activities in induced lysates of four strains of M type 49 streptococci were immunologically indistinguishable but different from the enzyme in induced lysates of a heterologous type. The four M type 49 strains were from widely different

geographical or temporal sources and of different phage subtypes as determined by lytic patterns.

These findings were confirmed in studies that employed a functional assay of enzyme neutralization. An immunoglobulin preparation of antiserum against the purified enzyme as well as one against homologous purified whole phage neutralized the hyaluronidase activity produced by induction of the M type 49 strains and present either phage-bound or soluble in phage-free lysates. These immunoglobulin preparations had little effect on the hyaluronidase activities present in phage-lysates of other M types of group A streptococci.

Inhibition of propagation of temperate phages by antibody against the purified phage hyaluronidase paralleled the neutralization of phage-associated enzyme activity by this antibody, indicating that antibody to the purified enzyme can inhibit phage infection.

Antibody preparations against the purified phage-bound enzyme or against purified whole phage did not neutralize the extracellular hyaluronidase in the supernate of an uninduced culture of M type 4 streptococci. A human serum strongly inhibitory for the extracellular enzyme of this strain had little effect on the hyaluronidase activities of an induced lysate of this strain or on the purified phage enzyme from an M type 49 strain.

The results support the view that the hyaluronidases associated with the temperate bacteriophages from various M types of group A streptococci do not share common antigenic determinants but that an immunological specificity exists that parallels the serologic specificity of the M protein of the host strains.

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