THE RÔLE OF THE MUCOID POLYSACCHARIDE (HYALURONIC ACID) IN THE VIRULENCE OF GROUP A HEMOLYTIC STREPTOCOCCI

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While it is generally agreed that the capsular polysaccharide of hemolytic streptococci of Lancefield group C is an important virulence factor (1-3), the capsular polysaccharide of group A streptococci, although apparently identical with that of the group C streptococcus, has been considered only a minor factor in virulence (2-4). The latter conclusion is based chiefly on evidence presented by Hirst (2), who infected mice with group A and group C streptococci and treated these animals with leech extract containing the specific decapsulating and hydrolyzing enzyme, hyaluronidase. Such treatment protected against the group C strain but gave only feeble protection against the group A organisms. Similarly, Blundell (5) found that mice infected with a group A strain and treated with bovine testicular extract showed a greater mean survival time than the untreated controls, but the protective action was weak. Attempts by McClean, however, (6) to repeat the observations of Hirst and of Blundell using testicular hyaluronidase have been unsuccessful, even with group C organisms, despite the use of amounts of enzyme greatly in excess of those necessary to bring about decapsulation in vitro.

The consistent presence of hyaluronic acid in group A streptococci isolated from human infections (7) and its capsular location, indicate the likelihood that the polysaccharide is more important than would appear from these observations.

Many earlier workers have suggested a basic rôle in virulence for the capsule. Bordet (8), in 1897, related the infectivity of streptococci to an "areola" which is present particularly in young streptococci, and which enables the organisms to resist phagocytosis, and Hare (11) demonstrated that as virulent streptococci age, they are more readily phagocytosed. That young streptococci are encapsulated was confirmed by Seastone (9), who also showed that removal of the capsules by aging or heat treatment is associated with increasing susceptibility to phagocytosis. Kendall, Heidelberger, and Dawson (10) isolated the capsular carbohydrate and indicated its probable identity with hyaluronic acid, a polysaccharide first found by Meyer and Palmer (12) in bovine vitreous humor and human umbilical cord. Hyaluronic acid has since been obtained from such diverse sources as skin (13), synovial fluid (14), fowl tumors (15, 16), a human mesothelioma (17), and from group C streptococci (1). Attempts to
produce an antibody to the polysaccharide have so far been unsuccessful, probably because the polysaccharide is a natural constituent of mammalian tissues.

Enzymes capable of hydrolyzing hyaluronic acid have been obtained from pneumococcal autolysates (18), testicular extract (19, 20), spleen (21), some non-mucoid hemolytic streptococci (21, 22), Clostridium welchii cultures (23, 24), snake and spider venoms (25), leeches (26), and from extracts of various tumors (16); hyaluronidase has been identified as one of the spreading factors (4, 19, 27).

The present studies are concerned with the protective effect of hyaluronidase on group A streptococcal infection, in the belief that the demonstration of such protection would establish the capsule as one of the basic factors in the virulence of group A streptococci.

Materials and Methods

Media and Cultures.—The stock medium used throughout was a 2 per cent bacto-tryptose, 1 per cent dextrose solution (pH 7.6–7.8) with sterile sheep serum added as needed. For culturing the pneumococcal strain used in these experiments, 1 per cent sheep serum broth was used; for the streptococcal strains, the serum was added to 20 per cent concentration. The pneumococci were maintained in meat tubes under mineral oil, whereas the streptococci were transferred monthly on 5 per cent sheep blood agar plates; all strains were passed through mice frequently to maintain virulence.

Preparation of Hyaluronic Acid.—The method of purification of vitreous humor polysaccharide was that of Seastone (1). The umbilical cords used as a source of hyaluronic acid were collected under acetone, minced with saline in a Waring blender, and the extract purified by the same method.

Preparation of Hyaluronidase.—Freshly obtained bull testicles were stripped free of epididymis and fibrous connective tissue coverings, and minced in a Waring blender with an equal weight of distilled water. The suspension was centrifuged and the turbid, red-brown supernatant brought to pH 4.5 (glass electrode) with 2 N acetic acid. After 1 hour in the refrigerator, a large, essentially inactive precipitate settled out, leaving a clear red or amber supernatant which was neutralized with 5 N NaOH, centrifuged, and filtered through a bacteria-retaining sintered glass filter. The solution can be either dried by lyophilization, or preserved in the frozen state in the refrigerator. The frozen solutions proved stable, with less than 20 per cent loss in activity after storage for 2 months.

Estimation of Hyaluronidase Activity.—Because of the inconvenience of the viscosi-metric method of McLean and Hale (23), a procedure was developed for measuring hyaluronidase activity by turbidimetric means. Since the turbidity produced when hyaluronic acid is mixed with acidified protein is a function of the concentration of the polysaccharide (1), reference turbidity curves may be plotted by adding known amounts of purified hyaluronic acid to acidified protein under standard conditions. The acidified protein solution is prepared by diluting horse serum 1:10 with 0.5 M acetate buffer (pH 4.2), and adding 4 M HCl until pH 3.1 is reached (7). In all experiments, the standard method used for turbidimetric measurements consisted of adding...
the polysaccharide (usually contained in a volume not exceeding 1 ml.) to sufficient
0.5 ~r acetate buffer (pH 4.2) to bring the final volume to 4 ml. To this solution is
added 1 ml. of the acidified serum; the turbidity is allowed to develop for 30 minutes
and is determined in a Klett-Duboscq photoelectric colorimeter using various thick-
nesses of plate glass for standards. This turbidity estimation may also be utilized as
a rapid means for following the purification of the polysaccharide, since, if a given
weight of a sample fails to produce as much turbidity as the same amount of highly
purified polysaccharide, it is evident that not all of the weight of the former sample is
hyaluronic acid.

For the estimation of hyaluronidase activity, the enzyme is diluted with pH 6.0
buffer (0.1 ~) and allowed to warm for 5 minutes in a 37°C. water bath. Purified
polysaccharide, diluted in the same buffer to a concentration of 0.4 mg./ml. is warmed
at the same time, and equal amounts of polysaccharide and enzyme solutions are
mixed and incubated. At regular intervals 1 ml. samples are removed from the reacting
mixture and added to a tube containing 3 ml. acetate buffer (pH 4.2) and 1 ml.
acidified serum; after 30 minutes at room temperature, the turbidity is read as pre-
viosely outlined. One turbidity-reducing unit has been arbitrarily defined as that
amount of enzyme which in 30 minutes will reduce the turbidity produced by 0.2 mg.
of hyaluronic acid to the equivalent of the turbidity produced by 0.1 mg. The tur-
bidity equivalent to that produced by half the initial amount of polysaccharide is the
half-turbidity point.

As turbidity forms in the presence of the enzyme, the latter may continue to act on
the polysaccharide and decrease the final turbidity. Many other factors may, how-
ever, combine to decrease the activity of the enzyme after sampling; the dilution
which the reacting mixtures undergo upon being added to four volumes of buffer and
serial, as well as the change to a less favorable pH, tend to slow the reaction rate.
Probably an even more important inhibitory factor is the effect of the polysaccharide-
protein combination. Preliminary experiments showed that approximately ten times
more enzyme is required to reduce the turbidity of a preformed polysaccharide-protein
precipitate, than to reduce the capacity of the polysaccharide alone to precipitate acid
protein.

In order that the turbidity readings be accurate, the enzyme contained in the sample
must have no appreciable effect on the formation and subsequent stability of the pre-
cipitate during the time which elapses between sampling and reading the turbidity.
In a series of duplicate experiments, the standard amount of polysaccharide was incu-
bated with 1, 2, and 3 units of hyaluronidase, and samples were removed every 5
minutes until the half-turbidity point had been passed. The turbidity formed by each
sample was read exactly 2½, 1, 2, and 18 hours after sampling. The turbidity produced
by samples from the tubes containing 1 and 2 units of enzyme was unaffected by 2
hours standing at room temperature, although about 20 per cent decrease occurred
during the 18 hour period; the turbidity produced by samples from the tube containing
3 units was unaffected by 1 hour standing, but showed a 15 per cent decrease after 2
hours. From these experiments it is seen that, if the half-turbidity point is attained
in less than 15 minutes, i.e., if more than 2 units are present, the readings must be made
within an hour to be accurate. It would, however, be more precise to accept as valid
only those readings in which the half-turbidity point is attained within a period of
15 to 45 minutes. If the half-point falls within this time range, the tubes in which the turbidity has formed may stand up to 2 hours without affecting the readings. Thus, the entire enzyme estimation procedure can be conducted without halting to read the turbidity formed by the first samples, and all tubes can be read after a period of 30 minutes following the withdrawal of the last sample, as long as the total time between the initial mixing of enzyme and polysaccharide and the time when the last turbidity reading is taken does not exceed 2 hours. Customarily, the procedure has been to withdraw samples 15, 25, 30, 35, and 45 minutes after the time of mixing.

In cases where there is no previous indication of the approximate concentration of enzyme in a given solution, samples should also be taken at 5 and 10 minute intervals, for, if the half-turbidity point should be reached rapidly, extrapolation will indicate the approximate dilution of enzyme which will bring the half-point into the more accurate 15 to 45 minute range. If the half-point should fall in the 15 to 45 minute range, but not at 30 minutes, the unitage is calculated by a method similar to that used by McClean and Hale for the viscosimetric method; a graph of the turbidity against time will indicate the time at which half-turbidity occurred. However, a simple extrapolation on the assumption of a straight line reduction in turbidity with time gives results very close to those obtained by the use of the graph. The calculation, when the time for half-turbidity to be attained has been determined, is the following:

\[
\text{Turbidity-reducing units} = \frac{\text{Standard time (30 min.)} \times \text{reciprocal of the dilution of enzyme}}{\text{Time for half-turbidity to be attained}}
\]

Thus, if a 1 to 60 dilution of the enzyme brought about half-turbidity in 40 minutes, the enzyme solution contains 45 turbidity-reducing units.

The turbidity reduction method for estimating hyaluronidase activity was compared with the viscosimetric method of McClean and Hale, and two turbidity-reducing units were found equal to one viscosity-reducing unit. The turbidity method eliminates the use of the viscometer with its attendant expenditure of time in cleaning, measurement, etc., and is more economical of materials than the viscosity method, since much less substrate is required to give measurable turbidity than to produce enough viscosity to allow accurate measurement of the half-point. The chief disadvantage of the turbidity method is its dependence upon purified hyaluronic acid for a substrate. Since the concentration of polysaccharide does not alter the time in which the half-viscosity point is reached, crude substrate solutions may be used in the viscosity method; in the turbidity method, on the other hand, changes in the concentration of substrate will affect the results, hence crude solutions of hyaluronic acid to be used for a substrate must first be adjusted so that 0.5 ml. produces the same degree of turbidity as is produced by 0.2 mg. of polysaccharide. Such solutions have been used to show the probable enzyme concentration of a given hyaluronidase preparation; in three crude polysaccharide preparations so tested, determinations of the concentration of hyaluronidase agreed within 10 per cent with determinations using purified substrate. Since insufficient numbers of samples have been tested to determine the accuracy with which the turbidity reduction method can utilize crude hyaluronic acid, its use must, at present, be confined to preliminary studies which will show the probable enzyme content of an unknown solution; the final determination should be repeated with purified hyaluronic acid and the probable enzyme dilution.
The use of crude polysaccharide to establish the approximate enzyme concentration will allow 500 to 600 determinations to be made from 250 mg. of purified hyaluronic acid. Further study of the effect of the contaminating substances in the crude polysaccharide solutions may indicate conditions under which the crude substrate may be utilized more accurately.

The viscosimetric measurements of hyaluronidase activity followed the procedure of McClean and Hale (23), using crude umbilical cord polysaccharide, prepared as before, but with the chloroform-butyl alcohol shakings halted before all the protein had been removed. The alcohol-precipitated polysaccharide was dried in vacuo and dissolved in pH 6.0 phosphate buffer (0.1 M) to such concentration that the solution had a relative viscosity of about 4 at 37°C. 1 ml. of this solution was warmed in an Ostwald viscometer for 5 minutes at 37°C, and an equal amount of similarly warmed enzyme dilution (in the same buffer) was added. The solutions were mixed by blowing and their viscosities measured. The foregoing method was calibrated against that of McClean and Hale (who used citrate buffer at 34°C.) in order to obtain a conversion factor which would eliminate the troublesome use of 34°C. Division by this factor, 1.1, was used to convert the values obtained in subsequent studies at 37°C. to units corresponding to those of McClean and Hale.

EXPERIMENTAL

Experiment 1. Effect of Testicular Extract on Phagocytosis of Group A and Group C Streptococci

Earlier experiments (9) in which loss of capsules was accompanied by increased susceptibility to phagocytosis are open to the criticism that the changes undergone by the streptococci as a result of aging or heat treatment could conceivably explain the increased phagocytosis. To test conclusively for an antiphagocytic effect of the capsule, young microorganisms are desirable, since they more closely approximate the physiologic state of bacteria during active infection. Preliminary studies indicated that the viability of streptococci is not diminished by exposure to testicular extract; and, since hyaluronidase will rapidly decapsulate groups A and C streptococci, the enzyme was utilized in the following experiments to demonstrate the antiphagocytic effect of the capsules of these organisms.

The procedure for studying phagocytosis was a modification of the whole blood method of Lyons and Ward (28); the organisms were grown in 20 per cent serum broth by adding a 1:10 inoculum from an 18 hour culture to previously warmed and aerated medium. After incubation for 1 hour, the cells were thrown down and suspended in 0.1 volume of normal salt solution; the turbidity of the resulting suspension lies between tubes 1 and 2 in the McFarland scale. Each of a series of small serological tubes received 0.5 ml. of freshly defibrinated human blood, 0.05 ml. of the organism, and 0.1 ml. of the appropriate enzyme solution or of saline. The tubes were stoppered with paraffined corks and rotated at 37°C. for 15 minutes in a rotator making two revolutions per minute. Smears were made from the tubes and stained with Wright's stain; the number of cocci ingested by 50 or 100 polymorphonuclear leukocytes was deter-
mained, as well as the per cent of active leukocytes. The same procedure was followed in the control experiments with the Type I pneumococcus, except that the cultures were grown for 4 to 6 hours before use, and, in one series of experiments, a drop of 1:100 dilution of the homologous rabbit antiserum was added to each tube to aid phagocytosis.

During the early studies, the organisms were grown in broth containing the enzyme, or else were centrifuged, treated with the enzyme, and washed before they were added to the blood. Later experiments showed that the addition of testicular extract directly to the blood has no apparent inhibitory effect on the phagocytes, and in all subsequent work the enzyme was added directly to the blood and organisms without previously exposing the bacteria to the decapsulating agent.

The results of a typical experiment are recorded in Table I; blood EHK had practically no opsonic activity for the group A, type 14 strain (S 23), but ingestion of streptococci was greatly increased upon addition of testicular extract.

<table>
<thead>
<tr>
<th>Blood</th>
<th>No. of cocci ingested by 100 leukocytes</th>
<th>Active leukocytes per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>CVS</td>
<td>258</td>
<td>37</td>
</tr>
<tr>
<td>CVS + testicular extract</td>
<td>1289</td>
<td>92</td>
</tr>
<tr>
<td>EHK</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>EHK + testicular extract</td>
<td>1346</td>
<td>92</td>
</tr>
</tbody>
</table>

The experiments demonstrate that the removal of the capsule by testicular extract greatly increases the rate of phagocytosis of groups A and C streptococci. The effect seems to be specific, since testicular extract does not affect the phagocytosis of sensitized or unsensitized pneumococci.
Experiment 2. Effect of Testicular Extract on the Bactericidal Action of Defibrinated Human Blood on Group A Streptococci, Type 14 (S 23)

In order to learn whether the increased phagocytosis of strain S 23 in the presence of testicular extract will lead to killing of the ingested organisms, bactericidal experiments were carried out in the same way as were the phagocytosis experiments, except that each tube received one drop of the appropriate dilution in broth of an 18 hour culture of the streptococcus. The tubes were rotated for 6 hours, washed into Petri dishes, and agar added. Typical results, presented in Table II, demonstrate the enhancement of the bactericidal activity of whole blood by testicular extract; control experiments using sensitized and unsensitized Type I pneumococci gave no evidence of increased killing as a function of the added testicular extract. The regularly occur-

TABLE II
Bactericidal Effect of Normal (EHK) and Immune (CVS) Human Blood on Group A Streptococcus (S 23) in Presence of Testicular Extract

<table>
<thead>
<tr>
<th>Blood</th>
<th>No. of organisms added in one drop</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100,000</td>
</tr>
<tr>
<td>CVS</td>
<td></td>
</tr>
<tr>
<td>CVS + testicular extract</td>
<td>41</td>
</tr>
<tr>
<td>EHK</td>
<td></td>
</tr>
<tr>
<td>EHK + testicular extract</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The symbol ~o indicates a plate on which colonies were too numerous to count.

ring inhibition of the killing power of immune blood in the presence of testicular extract suggests either a non-specific relative inhibition of phagocytic activity, or a more specific competition between the enzyme molecule and the opsonizing antibody for the surface of the bacteria.

Experiment 3. Protective Effect of Testicular Extract on Experimental Group A Streptococcus Infection in Mice

For reasons which will be discussed, the enzyme treatment used by other workers (2, 5, 6) in their unsuccessful attempts to protect mice against group A streptococcal infection, appeared to be inadequate; experiments were therefore performed in which more intensive enzyme treatment was administered.

Groups of Swiss mice were injected intraperitoneally with appropriate dilutions of a 3½ hour culture of the test organism, grown from a 1:10 inoculum from an 18 hour culture. The final dilutions were made in appropriate amounts of testicular extract and allowed to remain at room temperature for 5 minutes before injection; organisms intended for the control mice were diluted in plain broth. Plate counts were made of
the dilutions injected. In a typical experiment, 0.4 ml. of the culture dilutions were injected and treatment begun 2 hours later with 0.5 ml. of the crude testicular extract containing 200 turbidity-reducing units or 100 viscosity-reducing units of hyaluronidase. The therapeutic injections, administered intraperitoneally, were given every 2 hours for the first 12 hours, every 4 hours for the next 36 hours, and every 12 hours for the last 48 hours.

The results of typical experiments are tabulated in Tables III, IV, and V. It is noteworthy that in all these experiments, young, physiologically active bacteria were injected, whereas in the unsuccessful experiments of other workers

**TABLE III**

*Protective Effect of Bovine Testicular Extract on Mice Infected with Group A Streptococci, Type 14 (S 23)*

<table>
<thead>
<tr>
<th>Dilution of culture</th>
<th>Virulence controls</th>
<th>Treated with testicular extract</th>
<th>Treated with testicular extract heated at 60°C. for 30 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mouse No.</td>
<td>10^-4</td>
<td>10^-3</td>
</tr>
<tr>
<td>1</td>
<td>D1</td>
<td>D1</td>
<td>D2</td>
</tr>
<tr>
<td>2</td>
<td>D1</td>
<td>D2</td>
<td>D2</td>
</tr>
<tr>
<td>3</td>
<td>D1</td>
<td>D2</td>
<td>D2</td>
</tr>
<tr>
<td>4</td>
<td>D1</td>
<td>D2</td>
<td>D2</td>
</tr>
<tr>
<td>5</td>
<td>D1</td>
<td>D3</td>
<td>D2</td>
</tr>
<tr>
<td>6</td>
<td>D1</td>
<td>D4</td>
<td>D2</td>
</tr>
<tr>
<td>7</td>
<td>D1</td>
<td>S</td>
<td>D2</td>
</tr>
<tr>
<td>8</td>
<td>D1</td>
<td>S</td>
<td>S</td>
</tr>
</tbody>
</table>

D, with a number, indicates death within that number of days. S means survival throughout the time of experiment, 14 days.

18 hour cultures were used. Presumably infection would be established more rapidly by the former method which therefore constitutes a more severe test of the therapeutic agent. In Table III, the results of an early experiment using type 14, group A streptococcal strain S 23 are presented; the colony count of the 10^-5 dilution in this experiment was 68 colonies in 0.4 ml. These experiments have been repeated with different preparations of the enzyme with essentially the same results. The protective action, which is effective against 10 to 100 M.L.D., is destroyed by heating at 60°C. for 1 hour. Since Hirst (2) failed to demonstrate protection against strain D 58 (type 3) as well as S 23, we also tested the former strain. Table IV shows that protection was obtained, even though the 10^-4 dilution of the culture used contained 80 colonies (more than 10 M.L.D.) in the infecting dose. The experiment tabulated in
Table V shows that the protection is specific; in this the Type I pneumococcus was used as the infecting agent and treatment was carried out with the enzyme.

### TABLE IV

**Protective Effect of Bovine Testicular Extract on Mice Infected with Group A Streptococci, Type 3 (DSg)**

<table>
<thead>
<tr>
<th>Mouse No.</th>
<th>Virulence controls</th>
<th>Treated with testicular extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dilution of culture</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$10^{-4}$</td>
<td>$10^{-5}$</td>
</tr>
<tr>
<td>1</td>
<td>D1</td>
<td>D1</td>
</tr>
<tr>
<td>2</td>
<td>D1</td>
<td>D1</td>
</tr>
<tr>
<td>3</td>
<td>D1</td>
<td>D1</td>
</tr>
<tr>
<td>4</td>
<td>D1</td>
<td>D1</td>
</tr>
<tr>
<td>5</td>
<td>D1</td>
<td>D1</td>
</tr>
<tr>
<td>6</td>
<td>D1</td>
<td>D1</td>
</tr>
<tr>
<td>7</td>
<td>D1</td>
<td>D1</td>
</tr>
<tr>
<td>8</td>
<td>D1</td>
<td>D1</td>
</tr>
<tr>
<td>9</td>
<td>D1</td>
<td>D2</td>
</tr>
<tr>
<td>10</td>
<td>D1</td>
<td>D2</td>
</tr>
</tbody>
</table>

D, with a number, indicates death within that number of days. S means survival throughout the time of the experiment, 8 days.

### TABLE V

**Protective Effect of Bovine Testicular Extract on Mice Infected with Type I Pneumococcus**

<table>
<thead>
<tr>
<th>Mouse No.</th>
<th>Virulence controls</th>
<th>Treated with testicular extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dilution of culture</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$10^{-4}$</td>
<td>$10^{-5}$</td>
</tr>
<tr>
<td>1</td>
<td>D2</td>
<td>D1</td>
</tr>
<tr>
<td>2</td>
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<tr>
<td>3</td>
<td>D2</td>
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</tr>
<tr>
<td>4</td>
<td>D2</td>
<td>D2</td>
</tr>
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<td>5</td>
<td>D2</td>
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<td>6</td>
<td>D2</td>
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<tr>
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<td>D2</td>
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<tr>
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<tr>
<td>9</td>
<td>D2</td>
<td>D2</td>
</tr>
<tr>
<td>10</td>
<td>D2</td>
<td>S</td>
</tr>
</tbody>
</table>

D, with a number, indicates death within that number of days. S means survival throughout the time of experiment, 8 days.

preparation that had protected against strain D 58. The hyaluronidase not only failed to protect against the Type I pneumococcal infection, but seemed to hasten the fatal outcome; a study of the time of death of the mice strikingly confirms this point.
The consistent presence of hyaluronic acid in group A streptococci isolated from human infections (7) indicates the likelihood of a more important role for the polysaccharide than has been accepted by Hirst and Lancefield (2, 3); that the polysaccharide is a significant factor in virulence is demonstrated by the experiments reported in this paper. Lancefield has shown that the M protein, which is partly responsible for the type specificity of group A streptococci, will give rise to protective antisera; and Mudd et al., using old cultures which lacked capsules, showed that virulent strains of streptococci are less readily phagocytosed than non-virulent variants of the same strain (29). While it is thus apparent that factors other than hyaluronic acid contribute to the virulence of group A streptococci, the ability of hyaluronidase to promote phagocytosis and bactericidal activity, as well as to protect against infection in mice, shows that hyaluronic acid is fundamentally connected with virulence. The relation of the M substance to virulence remains an open question, since it has been obtained in equally large amounts from both virulent and avirulent strains (30). Furthermore, the production of protective antibodies does not necessarily depend upon an antigen closely related to virulence, for non-type-specific protection may be stimulated by rough, avirulent pneumococci (31). The possibility that hyaluronic acid and the M protein are associated in a virulent strain has been pointed out (7).

That protection against group C infection by hyaluronidase has been more readily attained than protection against group A streptococci may be explained by the observation that the group C infections under investigation develop more slowly, and produce a fatal termination anywhere from 12 to 24 hours later than do the more rapidly developing group A infections, possibly as an expression of different growth rates. It is thus reasonable to suppose that the defense mechanisms of the host have a certain initial advantage in the group C infections, so that a larger amount of enzyme is necessary to protect against the group A organisms.

Another reason for the failure of previous workers to protect against group A infection with hyaluronidase may be surmised from the work of McClean (6) who demonstrated that the sera of many animals, including mice, inhibit the activity of the enzyme. McClean studied the decapsulating effect of hyaluronidase in mice and pointed out that 100 viscosity-reducing units of enzyme maintained complete decapsulation for only about 1½ hours; yet in his attempts to demonstrate protection against streptococcal infection, he used but 10 such units and injected them at 8 hour intervals, in accordance with the procedure of Hirst. The absence of protection in such an experiment may well be explained on a purely quantitative basis. McClean (32) has suggested that his failure to protect may be related to the high state of purity of the enzyme.
preparation which he used. Highly purified hyaluronidase was more rapidly removed from the blood stream than a crude preparation of the enzyme.

The inhibition of the bactericidal effect of immune whole blood by hyaluronidase is difficult to explain. While the high concentration of hyaluronidase might suggest a non-specific inhibition of the bactericidal system by the enzyme, the fact that the enzyme greatly increases the killing power of non-immune blood seems to contradict such an explanation. Another possible explanation is that the anti-M opsonin and hyaluronidase compete for the surface of the streptococcal cell, so that the subsequent steric hindrance between the two molecules in some way decreases the ultimate phagocytic response. Such an explanation is based on the assumption that the M protein is at or near the capsular surface, an assumption which is borne out by the fact that anti-M antibody opsonizes encapsulated streptococci, and by the observation of Lancefield (3) that streptococci treated with trypsin may lose their type specificity without apparent loss of any vital function.

SUMMARY

1. A quantitative turbidimetric method for the estimation of hyaluronidase activity, based on the ability of the enzyme to decrease the capacity of the polysaccharide to precipitate acidified protein has been developed. Two units of hyaluronidase, by this method, are equivalent to one viscosity-reducing unit.

2. Hyaluronidase added to a phagocytic system containing defibrinated human blood, immune or non-immune, greatly increases the rate of phagocytosis of group A streptococci. Phagocytosis of Type I pneumococci is not affected by hyaluronidase under the same conditions.

3. The bactericidal activity of non-immune blood against group A streptococci is increased by hyaluronidase; the activity of immune blood is, however, somewhat inhibited by the enzyme. Killing of pneumococci is not affected by the presence of the enzyme.

4. Mice can be protected against group A streptococcal infection by frequent treatment with 200 turbidity-reducing units of hyaluronidase; the protective action of the enzyme is removed by heating at 60°C. for 1 hour. Mice infected with Type I pneumococcus and treated with hyaluronidase die somewhat sooner than the untreated controls.

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