THE OCCURRENCE OF MUCOID POLYSACCHARIDE IN HEMOLYTIC STREPTOCOCCI OF HUMAN ORIGIN

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It has been shown by Kendall, Heidelberger, and Dawson (1) that hyaluronic acid is one of the main components of the hemolytic streptococcus capsule. This polysaccharide was originally described by Meyer and Palmer (2, 3) in vitreous humor, umbilical cord, and synovial fluid, and it has since been isolated from other sources, namely skin (4, 5), a human mesothelioma (6), and the Rous sarcoma (7). Attempts to produce antibodies against this substance have so far been unsuccessful and there would seem to be little possibility that specific serologic reactions can be used for its identification.

Hyaluronic acid will unite with normal serum proteins and egg albumin at about pH 4 to produce an insoluble complex. This property originally noted by Meyer and Palmer (2) has been employed in a variety of ways (8, 9) to estimate the material; it can exclude the presence of as little as 2 gamma per cc. with certainty, although it is obviously not a specific reaction.

Enzymes capable of hydrolyzing hyaluronic acid, incidentally destroying the property of union with protein at pH 4, have been obtained from pneumococci (10, 11), *B. welchii* (10), testicular extract (12, 13), leech extract (4, 13), and from a few non-mucoid hemolytic streptococci (9, 13). Such enzymes used in conjunction with the protein reactions noted above will give a fairly accurate impression of the presence and amount of the polysaccharide in impure mixtures.

The purpose of the present study, based on the foregoing facts, was to determine the occurrence of hyaluronic acid, or mucoid polysaccharide, in a number of beta hemolytic streptococci obtained from human infections, and from normal throats in which no subsequent infection developed.

Materials and Methods

125 strains were investigated; of these, 90 were from the Wisconsin General Hospital and Student Infirmary,[1] obtained over a period from November, 1940, to May, 1941, these organisms presumably derived from infectious processes. 35 strains were from routine throat cultures of children admitted to the Children's Hospital at Iowa City.[1] In none of the latter group was any infection noted after admission.

In addition to the mucoid polysaccharide (henceforth referred to as MP) estima-

[1] We are indebted to the staff of the Wisconsin State Laboratory of Hygiene for its cooperation in obtaining the hospital strains, and to Miss Marion Jones, University of Iowa Medical School, for sending us the normal throat strains.

tions, the Lancefield group and encapsulation of each strain was determined. The clinical records of the patients furnishing the 90 hospital strains were examined to confirm as far as possible the presence of infection as well as its severity.

Isolation of Strains.—Pour blood plates were made with saline suspensions of the throat or wound swabs, and beta hemolytic colonies picked from these to streak blood plates to determine purity. This second plate was the source of organisms for the subsequent procedures, and except as noted, no further passage on artificial medium was carried out. The pour plate is desirable since 21 of the strains showed beta hemolysis only when under the surface of the agar, giving the alpha change on the surface. This was confirmed on the secondary streak plates by incising the agar in the heavy portion of the streak thus pushing the organisms beneath the surface. Under these conditions strong beta hemolysis was observed, as in the original plate. The majority of the non-group A strains exhibited this behavior, although 14 of the 21 strains were in group A. The 35 normal throat strains were from triplicate streak plates incubated aerobically, in 10 per cent CO_2, and anaerobically.

Method for Quantitating the Mucoid Polysaccharide.—In working out a method for determining mucoid polysaccharide (MP) based on the acid protein reaction, simplicity and speed were sought since the method was to be applied on a rather large scale. The material can be obtained either from neutralized 24 hour culture supernates, or from very young capsulated organisms. On heating such young organisms the polysaccharide is promptly released into the solution.

At first a one-tube method was investigated in which the turbidity developing in mixtures of culture supernates and acidified buffered serum was compared with $BaSO_4$ standards. Reproducible results could be achieved only with extraordinary attention to details of pH, salt concentration, and composition of the medium. Since it was felt that the method would not be generally practicable, it was abandoned.

Another method which was followed for a time was based on the observation that young capsulated organisms would agglutinate very vigorously when mixed with equal amounts of 0.5 M acetic acid. That the agglutination was very likely due to the interaction of MP and protein was indicated by the following where it appeared that the bacterial body was furnishing the protein component: 24 hour (spontaneously decapsulated) streptococci also agglutinated in 0.5 M acetic acid, but the reaction was abolished by 4 washings in distilled water; this did not occur with the young capsulated form. Resuspending the old washed organisms in either the original supernatant broth or in purified vitreous humor hyaluronic acid solution restored the agglutination. No reaction occurred in 0.5 M phosphate buffer at pH 7. The mechanism of the young capsulated organism flocculation was not immediately apparent since the surface exposed in this case would be preponderately polysaccharide. However it was found that under such conditions of acidity the MP is very rapidly released from the cell with an associated disappearance of the capsule, the situation then becoming similar to that of the old culture. A sufficient number of non-MP producing strains exhibited acid agglutination to invalidate any general application of this as a method.

The procedure finally adopted consisted of serial dilutions of neutralized culture supernatants layered over acidified normal serum, precipitation taking place at or above the interface. The medium employed was bacto-neopeptone 1 per cent, bacto-

beef extract 0.5 per cent, dextrose 1 per cent, NaCl 0.5 per cent, and sterile sheep serum 10 per cent, distributed in 5 cc. amounts in centrifuge tubes. Inoculations were made from the second blood plates noted above. After 24 hours' incubation at 37°C., 2 drops of 0.02 per cent phenol red were added and the culture was brought to about pH 7.6 with 0.5 M NaOH. This neutralization is essential since sufficient acid may be developed during growth to cause complete MP precipitation, either with the bacterial or serum proteins. After centrifugation of the neutralized culture, serial dilutions of the clear supernatant medium in physiologic salt solution were made, 1:10, 1:20, 1:40, and 1:80. These dilutions, as well as the undiluted supernatant, were run into micro precipitin tubes[2] over acidified normal horse serum, and readings made after ½ hour. The acid serum reagent was prepared in the following manner: Clear, non-hemolyzed horse serum was diluted 1:10 with 0.5 M acetate buffer at pH 4.2. The reaction was brought to pH 3.1 with 4 M HCl, and merthiolate 1:1,000 added to a final concentration of 1:100,000. It should be noted that colorimetric (brom cresol green) pH determinations in the presence of this amount of protein are extremely inaccurate, however, no significant difference in titre was noticed in a pH range between 3 and 4. Uninoculated medium gave no ring at the interface. The acidified serum may be stored in the cold for many months.

Cultures reacting in a dilution higher than 1:80 have not been encountered. Using purified hyaluronic acid from bovine vitreous humor, an end-point of comparable intensity is obtained with a solution containing 0.002 mg. per cc. This would indicate the presence of about 0.16 mg. per cc. of culture supernatant in a strain producing maximal amounts; a figure of the same order of magnitude has been found for group C strains by means of a somewhat more accurate method (8), as well as for group A strains by direct yield (14).

Evaluation of MP Estimation Method.—Although it is perhaps unlikely that any of the recognized streptococcal somatic elements would be liberated during growth in large enough concentration to precipitate under the conditions of this method, the question of its specificity naturally arises. It will be shown later that the majority of strains possessing acid serum reactivity fall into Lancefield's group A, which might point to the "C" substance as the responsible factor. With this possibility in mind, formamide extracts (15) were prepared with A strains giving 1:80 acid serum titres, and from A strains in which no reaction appeared. These extracts were tested with a potent A grouping serum, with the result that the latter non-MP producing strains reacted in slightly higher dilutions than did the former strong producers. Furthermore, neutralized culture supernates failed to give precipitin reactions in any dilution when tested with group A specific serum.

The M substance of Lancefield was also to be considered in the light of its known acid precipitability. The following findings tend to eliminate this and the C substance as sources of error. Neutralized (pH 6 in this case) culture supernates were prepared from 31 strains, including all degrees of acid serum reactivity. Treating these with hyaluronidase from a Type I pneumococcus (10, 11) either eliminated completely, or reduced to a faint trace, the acid serum precipitate in all but one of the

[2] About 1 inch long from tubing 3 to 4 mm. inside diameter. Such tubes are used only once.

31 tested. This one reacted in a dilution of 1:10 before and after enzyme treatment. From the foregoing it may be concluded that in almost all instances the estimation method given here is reasonably reliable.

Encapsulation.—Before neutralization of the 24 hour culture, 0.2 cc. were inoculated into 2.0 cc. of the medium described above in which 50 per cent defibrinated sheep blood was substituted for the serum. After incubation for 3½ hours at 37°C., Wright's stained films were prepared.

Lancefield Grouping.—The formamide micro method described by Fuller (15) was used on organisms from serum-free broth.

FINDINGS

In addition to MP determinations, Lancefield grouping, and capsule stains, the type of colony appearing on freshly prepared neopeptone blood agar as recommended by Dawson, Hobby, and Olmstead (16), and the nature of the growth in 10 per cent serum broth were noted. Streptococci failing to produce the MP factor almost invariably remained glossy. However, among the strains producing this material, many were encountered which also showed no disturbance of the colonial surface. Furthermore in those strains showing flattening, crater formation, or roughening of the surface, one could not correlate the degree of this change with the amount of MP produced.

Concerning the growth in 10 per cent serum broth, there was a distinct tendency for the non-producing strains to show an extremely granular type of growth, while the MP producers remained uniformly suspended. These observations were made on neutralized cultures to eliminate the effect of MP-protein precipitates. In our particular group of strains this could be regarded only as a tendency, the most common exception being the frequent occurrence of non-MP producing strains which grew as very uniform suspensions under these conditions.

The results appear in Table I from which several facts may be demonstrated. All the strains showing positive reactions in the 1:40 to 1:80 range of dilution may be shown to be capsulated. In the 1:20 range, capsules appear irregularly, and below 1:20 no stainable capsule could be found. In general, the largest capsules appeared in the 1:80 group but it was not always possible to correlate capsular size with MP titre. Different strains retain their capsules with varying degrees of tenacity, and exhibit different growth rates. It would therefore be unwise to assume that any one arbitrary incubation period would allow optimal capsulation of all strains. Of the 42 non-group A strains,[3] not one was found to produce detectable amounts of mucoid polysaccharide. The distribution of group A strains in the hospital compared with the normal throat strains is noteworthy, confirming the well established observation of Lancefield (17) that most streptococcal infections in man are due to this group. Of the

[3] These were in groups B and C with one exception which could not be classified.

35 strains from normal throats only 3 were MP producers, all of them capsulated and belonging to Lancefield's group A. No other group A strains were encountered here. In sharp contrast are the 90 hospital strains; if one disregards the 8 strains which were not associated with infection, 78 of 82 strains are group A, and 72 are MP producers.

In evaluating the severity of the infections it is fully recognized that the virulence of the organism is not the only factor involved. Of perhaps equal importance in affecting the clinical picture is the individual host resistance as well as the sulfonamide therapy which most of the patients received. In

TABLE I

Distribution of Mucoid Polysaccharide

Titre in acid serum	1:40 to 1:80	1:20	1:10	Undiluted or 0
90 hospital and infirmary strains				
No. of strains	51	15	6	18
Showing capsules	51	12	?1	?1
Lancefield group A	51	15	6	6
From severe infections	14	2	1*	0
From moderate infections	29	11	4	5
From mild or doubtful infections	8	2	1	5
Giving no clinical evidence of infection	0	0	0	8
35 normal throat strains				
No. of strains	2	1	0	32
Showing capsules	2	1		0
Lancefield group A	2	1		0

* Complicated by *Staphylococcus aureus.*

spite of these limitations the infections were grouped as severe, moderate, and mild. It was of interest that a certain number of the strains were derived from patients showing no clinical or pathological evidence of streptococcal infection.

The group of severe infections comprised those showing a septic febrile course, a positive blood culture, or a particularly protracted illness with or without a spiking temperature curve. All exhibited marked leukocytosis. Four of these infections were fatal, and one required amputation of a limb. In the moderate group were placed infections of rather shorter duration and less alarming nature. Most of these were acute sore throats with more or less intense pharyngeal injection, and a 3 to 5 day temperature elevation, often followed by otitis media. It is not certain that all of the so called mild cases were of streptococcal etiology. They were usually transitory sore throats with a 24 to 48 hour febrile period sometimes without leukocytosis or marked pharyngeal

inflammation. One of them was an otitis media in a diabetic child aged 7, who remained afebrile throughout the course of the illness.

As is shown in the table, there is a progressive decrease in the proportion of high MP-producing strains as one goes from the severe types of infection to the mild ones. No fatalities occurred due to strains precipitating in a titre of less than 1:40. Although the numbers were too small to have much significance, a relatively greater proportion of children were infected with organisms of low MP content.

DISCUSSION

The data presented above tend to confirm the impression that most serious human hemolytic streptococcal infections are due to organisms in a phase described by Dawson (16) as mucoid, and by Todd and Lancefield (18) as matt, these designations very likely being synonymous. The important factor in the matt designation has been the M substance, while the common factor indicated by Dawson and others as associated with mucoid strains is the mucoid polysaccharide, or hyaluronic acid, presumably a capsular substance. The latter has not been sought in any very large number of human strains, and it is not impossible that these two substances are invariably associated in any matt, or mucoid strain. The present work shows the wide distribution of the mucoid polysaccharide in streptococci from man, and the wide variation in the amounts appearing in different strains. In the absence of a more or less quantitative study, many of these strains, although producing appreciable amounts of polysaccharide, would not ordinarily be classified as mucoid, indeed they could not under the current description of this phase.

Lancefield has shown that the protein M substance is partly responsible for type specificity in group A streptococci, and for the protective effect of antisera and vaccines. There is evidence that the anti-M antibody brings about opsonization of capsulated organisms; this has been suggested as a typing method (19). These facts would constitute good evidence that the M substance is responsible for virulence were it not for the observation that this material may be obtained in equally large amounts from avirulent strains (20). That one might stimulate protective antibodies with a substance not itself responsible for virulence is conceivable, recalling the non-type specific immunity obtained with the pneumococcus (21).

The possibility that the mucoid polysaccharide may play a part in virulence is raised in the first place by its capsular location; the fact that capsular autolysis is associated with the appearance of spontaneous phagocytosis in normal blood is also suggestive (22). Hirst (14) has studied the effect of leech hyaluronidase on protection. He was able to show that this enzyme which causes rapid decapsulation in strains of either group A or C, is protective only for mice infected with group C strains, and it was concluded from this (23) that the

capsular substance plays little or no part in the virulence of group A strains. Using another source of hyaluronidase, an extract of beef testis, we have obtained essentially the same results with the same strains employed by Hirst, however it has been our experience that group C organisms of guinea pig origin are less rapidly fatal than are group A strains. The average time of death for the latter is around 24 hours, while the fatal period for group C infections is nearer 48 hours. With this in mind as a possible explanation for the differences in therapeutic effect, we repeated the experiment using larger amounts of enzyme at more frequent intervals, and obtained permanent protection in group A (S 23) infections involving between 10 and 100 M.L.D. This effect is mentioned only in a preliminary way for the purpose of discussion; more detailed study is in progress.

These findings point to the mucoid polysaccharide as a factor of definite significance in the virulence of hemolytic streptococci of human origin.

SUMMARY

1. A rapid method for the roughly quantitative estimation of mucoid polysaccharide in hemolytic streptococci has been described.

2. Using this method, about 94 per cent of strains from moderate or severe streptococcal infections in man have been found to produce mucoid polysaccharide in greater or less amount. In a group of streptococci from normal throats only about 8 per cent produced this substance, all of the producers falling into Lancefield's group A.

3. Of the Lancefield group A strains from both normal and infected sources, 92 per cent showed the presence of mucoid polysaccharide in culture dilutions of 1:10 or higher.

4. The probable significance of the mucoid polysaccharide in streptococcal virulence is indicated.

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