STUDIES ON THE HEMOLYTIC STREPTOCOCCUS OF HUMAN ORIGIN

II. Observations on the Protective Mechanism against the Virulent Variants

By CHAMP LYONS,* M.D., and HUGH K. WARD, M.B.

(From the Department of Bacteriology and Immunology of The Harvard Medical School, Boston)

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In the preceding paper we have described the characteristics of two human-virulent variants (F and M) of the hemolytic streptococcus, and have presented the evidence which leads us to believe that the determining factor in the virulence of these two variants is their capacity to resist phagocytosis, although the exotoxin, the fibrinolysin of Tillett and Garner (1), and the delay in inflammatory fixation described by Menkin (2) may well be important accessory factors in human infections.

Were this conception of virulence correct, it should be possible to demonstrate opsonizing antibodies in the serum of the immune animal. Such opsonins were demonstrated by Denys and Leclef (3) in 1895, and by many other workers since that time. The difficulties of the phagocytic technique have discouraged extended studies of the opsonic antibody. In the accompanying paper we described a method of demonstrating the resistance to phagocytosis of streptococci in infant's blood; *i.e.*, in the absence of specific opsonin. In the present paper we are reporting the phagocytosis induced by adding opsonin to such a system, with studies upon the specificity and identity of the opsonin with other known antibodies to the streptococcus. The word "opsonin" is used throughout to connote that specific heat-stable antibody which combines with the organism to render it susceptible to phagocytosis; no distinction is drawn between the so called natural

* Fellow in Medicine, National Research Council.

opsonins, immune opsonins, and bacteriotropins, inasmuch as we believe they are qualitatively identical (4).

It has long been recognized that the streptococci form an antigenically heterologous group, and many attempts have been made to type these organisms with antibacterial sera. The majority of workers have used agglutination, but, owing to the multiplicity of antigens involved, have met with many difficulties (5). Dochez, Avery, and Lancefield (6) in 1919 used agglutination and mouse protection in typing strains obtained from an epidemic of bronchopneumonia. On this basis, they were able to divide two-thirds of their strains into four groups, the remaining third being unclassified. More recently, Lancefield (7) has isolated the type-specific precipitinogen of the streptococci of human origin, designating it the M substance.

Since both the mouse protection and precipitin tests, although accurate, present considerable practical difficulties, as will be emphasized later, we have investigated the possibility of typing the streptococci by the opsonic technique. We have further attempted to identify the opsonin with the type-specific antibody which precipitates the M substance of Lancefield (7) and to differentiate the opsonin from the specific agglutinating antibody.

Experimental Procedures

(*a*) *Source of Material.*—The same as in Paper I.

(*b*) *Cultivation.*—The same as in Paper I.

(*c*) *Phagocytosis in Human Blood.*—The same technique was used as in Paper I, save that in experiments where the opsonins in immune sera were being studied 1 drop (0.03 cc.) of diluted serum was added to the system. We have found that foreign serum in strong concentration inhibits the phagocytosis of the streptococcus in human blood. Consequently, we have always diluted rabbit serum 1:10 and horse serum 1:5 before adding to the phagocytic system.

(*d*) *Preparation of Antisera.*—It has been the general experience of workers in this field that protective antisera are much more difficult to prepare than agglutinating antisera, and that living cultures must be used in the course of the immunization. This has also been the experience of Lancefield (7) in preparing sera which would precipitate the M substance. We have met with the same difficulty ourselves in preparing sera which contained the specific opsonins. This, indeed, has been our major handicap in attempting to type the virulent streptococci by the opsonic method. We have been unable to obtain opsonizing antisera by injecting cultures killed by heat, formalin, or alcohol. Even the injection of living cultures has been successful in only a few instances. Unfortunately, we realized too late the difficulties inherent in the production of these antisera, and there was then no time to

subject animals to the prolonged course of immunization which is apparently necessary. The method employed to produce active antiserum was as follows: 0.1 cc. of a 3 hour culture of the M variant in 20 per cent rabbit serum neopeptone water was injected intravenously into rabbits at weekly intervals. It was observed that the rabbits, in almost all cases, had a positive blood culture for from 1 to 3 days after this injection. In many rabbits we were unable to demonstrate the development of opsonins even after many weeks, during which this phenomenon had been repeatedly observed. We have not used the living F variant as an antigen.

(*e*) *Preparation of Bacterial Extracts.*—Extracts were prepared from 16 hour broth cultures of the F and M variants exactly as described by Lancefield (7).

(*f*) *Absorption of Antisera.*—All absorptions were carried out according to the technique of Lancefield (7), 16 hour broth cultures of the F and M variants being used.

(*g*) *Precipitation Tests.*—The bacterial extracts were layered on the unabsorbed and absorbed antisera and read after 2 hours at room temperature. Some of our antisera gave a positive ring test with broth or peptone alone. To avoid error from this source, it is necessary to wash thoroughly cultures which are to be used for the preparation of bacterial extracts. This phenomenon made it impossible in most cases to investigate any bacterial precipitinogens in the supernatant fluid of broth cultures.

(*h*) *Agglutination Tests.*—We have used two techniques in preparing cultures for agglutination tests: (1) Young cultures of the M and F variants in 50 per cent horse serum neopeptone water were prepared as for the phagocytic test. (2) Cultures of the M and F variants were grown in 5 per cent horse serum neopeptone water for 15 hours after the first visible sign of growth.

In both cases, the cultures were well shaken up and 0.5 cc. added to an equal quantity of saline dilutions of the antiserum in agglutination tubes, the control tube for spontaneous agglutination containing culture and saline alone. These tubes were placed in the water bath at 55°C., and read at the end of 3 hours.

(*i*) *Bactericidal Technique.*—These tests were carried out by the modified Todd technique described by one of us (8). 16 hour cultures of the F and M variant in 5 per cent horse serum neopeptone water were serially diluted in neopeptone water, and added to the mixture of blood and diluted antiserum. To a control series, the same dilution of normal serum was added. The tubes were sealed, rotated in a 37°C. incubator for 48 hours, and then 1 drop of the contents of each tube plated out.

(*j*) *Passive Mouse Protection.*—A series of six mice were injected intraperitoneally with 0.2 cc. of a homologous antiserum, another series with 0.2 cc. of a heterologous antiserum, and a third series with 0.2 cc. of normal rabbit serum. The next day, each series was injected intraperitoneally with the living, virulent culture, one mouse receiving 0.5 cc. of the culture diluted 1:10, the next mouse receiving 0.5 cc. of a 1:100 dilution and so on, the sixth mouse in the series receiv-

ing 0.5 cc. of a 10^{-6} dilution of the culture. Mice which died before the end of the 4th day were autopsied and cultures made of the heart's blood. Mice which survived 4 days were assumed to be protected.

(*k*) *Phagocytosis in Mouse Protection Test.*—Phagocytosis in infant's blood of a young culture of the organism in the presence of the homologous antiserum, of the heterologous antiserum, and of the normal serum were carried out in the usual way. In addition, one mouse was injected intraperitoneally with 0.2 cc. of a homologous antiserum, and one mouse with 0.2 cc. of a heterologous antiserum; the next morning, each mouse received an intraperitoneal injection of 0.5 cc. saline, and 4 hours later an intraperitoneal injection of 0.01 cc. of a young culture of the organism grown in 50 per cent horse serum neopeptone water, centrifuged and resuspended in neopeptone water. The mice were killed 4 hours later; the peritoneal contents smeared on a glass slide, and stained with Wright's stain.

RESULTS

(*a*) *The Correlation between Mouse Protection and Phagocytosis.*—It is well known that mice can be protected against virulent hemolytic streptococci (M variant) by a previous injection of a specific antiserum. If this protection is due, or mainly due to the phagocytosis of the injected organisms, it is necessary to show that the protecting antiserum contains specific opsonin, and that phagocytosis actually occurs in the mouse in the presence of this serum.

Tables I and II show clearly that Antiserum 301 contains opsonin for the Strain S_{23} and protects mice against this strain, whereas Antiserum 190 and normal rabbit serum neither protect the mice nor opsonize the organisms.

If the peritoneal fluid of the mouse is studied 4 hours after 0.01 cc. of a young culture is injected, we have observed that in the case of the mouse receiving the specific antiserum, free cocci either cannot be found or are few in number, whereas many of the cells contain cocci, some of which stain faintly, suggesting that digestion has already commenced. On the other hand, in the fluid of the mouse receiving the heterologous antiserum, there are an enormous number of free, encapsulated streptococci, with no evidence of phagocytosis.

(*b*) *The Opsonic Action of Anti-M Sera on F and M Variants.*—We had only two opsonizing antisera for strains of which we had both the F and M variants. The opsonic experiments performed with these two antisera on the F and M variants of homologous and heterologous strains are recorded in Table III. In this experiment, Anti-

serum 189 was prepared by injecting a rabbit with living Phil. (M) culture and Antiserum 190 was prepared by injecting a rabbit with living Men. (M) culture. The strain C. H. was found to be opsonically homologous with the strain Phil. and is included in the experiment to demonstrate this method of typing. The strain Col. was not opsonized by either antiserum.

TABLE I

Dose of streptococci Strain S_{28}—M variant	Fate of mice injected with		
	Antiserum 301	Antiserum 190	Normal serum
10^{-1}	D*	D	D
10^{-2}	S	D	D
10^{-3}	S	D	D
10^{-4}	S	D	D
10^{-5}	S	D	D
10^{-6}	S	D	S

* In this table, D = death of mouse, S = survival of mouse.

TABLE II

No. of streptococci (Strain S_{28}—M variant) phagocyted by infant's blood plus		
Antiserum 301	Antiserum 190	Normal serum
291–100*	0–0	0–0

* In this and subsequent tables, the first figure represents the number of organisms phagocyted by 25 polymorphs, the second figure the percentage of polymorphs which have phagocyted one or more organisms. Thus, 291–100 indicates that 291 streptococci were counted in 25 polymorphs, and that no empty cells were met with.

(*c*) *The Relationship of the Specific Opsonin, the Specific Precipitin, and the Specific Agglutinin in Anti-M Variant Sera.*—In the experiment reported in Tables IV and V, we have tested for the specific opsonin, the specific precipitin, and the specific agglutinin in an antiserum unabsorbed, absorbed with the homologous atypical F variant, and absorbed with the homologous M variant. The experiment is controlled by absorbing the same antiserum with heterologous F and M variants. The whole experiment, including the absorptions, was

repeated with essentially the same findings. Attention should be drawn to the use of an atypical F variant (see Paper I) in the homologous absorption. This was unavoidable, since we had no antiserum strong enough to use with a homologous typical F variant.

Assuming that the results would be the same had a homologous typical F variant been used, the experiment recorded in Tables IV and V shows that: (1) The homologous M variant absorbs completely the specific opsonin, specific precipitin, and the specific agglutinin from the antiserum for both itself and the F variant. (2) The homologous F variant absorbs the specific agglutinin, but leaves unabsorbed the major part of the specific precipitin and the specific opsonin. (3) Neither the heterologous F variant nor the heterologous M variant

TABLE III

Strain	Variant	No. of streptococci phagocyted by infant's blood plus		
		Normal serum	Antiserum 189	Antiserum 190
Phil.	F (atypical)	6–8	217–88	4–4
Phil.	M	2–4	355–100	2–4
CH.	F (atypical)	18–20	238–80	21–24
CH.	M	0–0	92–40	0–0
Men.	F	0–0	4–4	96–52
Men.	M	0–0	0–0	51–28
Col.	F	0–0	6–4	0–0
Col.	M	0–0	0–0	0–0

appreciably absorb the specific agglutinin, the specific precipitin, or the specific opsonin.

These findings suggest that: (1) The specific opsinogen is identical with all or part of the specific precipitinogen (the M substance of Lancefield (7)). (2) The specific agglutinogen is not antigenically identical with the specific precipitinogen or the specific opsinogen.

The previous experiment reported in Table III indicates that the F and M variants in young culture have the same opsinogen, and the only explanation we can offer for the failure of the F variant in an old culture to absorb the specific precipitin and the specific opsonin is that, in the old cultures used for absorption, the F variant has lost its specific opsinogen and specific precipitinogen, whereas the M

variant has retained this antigen or at least has retained it in part. Owing to the difficulty of centrifuging young cultures of the F variant, it has proved technically impossible to absorb serum with young F organisms.

TABLE IV

Serum	Agglutination							Precipitation
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	
Unabsorbed *vs.* M	++++	++++	++++	++++	+++	0	0	++++
" " F	++++	++++	++++	++++	++++	++	0	++
Homologous F-absorbed *vs.* M	0	0	0	0	0	0	0	+++
" " " F	+	0	0	0	0	0	0	+
Homologous M-absorbed " M	0	0	0	0	0	0	0	0
" " " F	0	0	0	0	0	0	0	0
Heterologous F-absorbed " M	++++	++++	++++	++++	++++	+	0	++++
" " " F	++++	++++	++++	++++	++++	++	+	+
Heterologous M-absorbed " M	++++	++++	++++	++++	0	0	0	++++
" " " F	++++	++++	++++	++++	++++	+	0	+

TABLE V

Serum	Phagocytosis of M variant*						
	Concentration of serum						
	1:80	1:160	1:320	1:640	1:1280	1:2560	0
Unabsorbed	1063–86†	636–64	270–30	177–18	38–8	20–4	10–2
Homologous F-absorbed	920–80	425–48	155–16	47–6	38–6	16–6	10–2
" M-absorbed	21–2	7–4	45–6	10–4	22–4	38–10	10–2
Heterologous F-absorbed	617–66	592–48	286–34	94–14	34–4	20–4	10–2
" M-absorbed	877–80	429–44	237–26	68–12	20–6	41–6	10–2

* In another experiment the F variant was used as well as the M variant, with essentially the same result.

† In this experiment 50 cells were counted instead of 25 cells.

The conception that the specific agglutinogen is antigenically different from the specific opsinogen, was further strengthened by the isolation of a strain whose mucous (M) variant was agglutinated to titer but not opsonized by an antiserum known to possess both specific agglutinin and specific opsonin for its homologous strain. This

agglutinative similarity and opsonic heterogeneity is graphically illustrated in Table VI.

A further observation is of interest in the study of the antigenic structure of the virulent variants of the hemolytic streptococcus. This is the inagglutinability of the F and M variants in that phase of growth in which they resist phagocytosis. Cultures grown for 3 hours in 50 per cent horse serum neopeptone water cannot be specifically agglutinated, whereas the same cultures grown for 15 hours after the first sign of visible growth in 5 per cent horse serum neopeptone water can be specifically agglutinated to a high titer. It can be shown that this inagglutinability of the young culture is not due to inhibition by the high percentage of horse serum in the culture media, since the young culture still resists agglutination when it is resuspended in saline. One might suppose that this phenomenon is attributable to

TABLE VI

Strain	Variant	Agglutinative titre in Antiserum 189	Phagocytosis in infant's blood plus		No. of organisms killed in human blood plus	
			Normal serum	Antiserum 189	Normal serum	Antiserum 189
Phil.	M	1:2560	0–0	225–96	19	19,000
845	M	1:2560	0–0	0–0	16	160

the masking of the somatic agglutinogen by the overlying capsular opsinogen, which is intact in young cultures, but apparently imperfect in older ones. The disappearance of the capsule in ageing cultures may be observed by staining methods and inferred from the increasing susceptibility to phagocytosis, as was pointed out by Seastone (9) and Hare (10). In this connection, we have seen capsular remnants in the old cultures of the M variant but not in those of the F variant, which may explain the absorption of the specific opsinogen and the specific precipitinogen by the M variant and the failure of the F variant to absorb this antibody.

We have no explanation, however, of our failure to demonstrate agglutination of the capsulated cultures by the opsonin, nor of our failure to demonstrate the *Quellung* phenomenon of Neufeld in these cultures, unless it be that our antisera contained too little of this antibody.

(*d*) *Opsonic Typing of the Hemolytic Streptococci.*—This phase of the work was severely handicapped by our difficulties in producing opsonizing antisera, but the five available sera indicated the immensity of the problem. One antiserum identified two homologous strains out of twenty-two examined; two antisera did not identify any strains out of twenty-four examined. Investigation of two commercial polyvalant antibacterial sera revealed no effective concen-

TABLE VII

Strain	Variant	Phagocytosis in the blood of					
		Infant	H.K.W.	C.L.	J.H.M.	F.B.G.	J.F.E.
Men.	F	0–0	2–4	69–28	0–0	122–72	14–16
"	M	0–0	0–0	0–0	0–0	16–16	0–0
W	"	0–0	104–72	0–0	0–0	0–0	0–0
S_{23}	"	0–0	34–32	2–4	12–8	126–76	—
SR	F	0–0	0–0	14–6	38–24	14–20	54–40
Cal.	"	0–0	0–0	41–24	4–8	2–4	155–60
FL	M	0–0	2–4	2–4	0–0	4–4	0–0

TABLE VIII

Strain	Variant	Infant's blood	Concentration of adult's serum	Phagocytosis
		cc.		
Phil.	M	0.25	1:8	147–84
"	"	0.25	1:16	52–72
"	"	0.25	1:32	40–32
"	"	0.25	1:64	21–20
"	"	0.25	1:128	5–4
"	"	0.25	—	0–0

tration of opsonins for any of forty-seven strains examined, thus confirming similar work by Hare (11) and by Fothergill and Lium (16).

(*e*) *Natural Opsonins.*—During the course of these experiments, an M variant was isolated from the throat of one of us during an attack of acute pharyngitis. Some months after convalescence, and long after the organism had disappeared from the nasopharynx, this individual's blood phagocyted this organism readily, whereas we were unable to demonstrate any phagocytosis of this organism by the blood

of any other adult in the laboratory. Seastone (9) has reported a similar experience, using essentially the same phagocytic technique. These observations would appear to explain the absence of opsonins to virulent streptococci in infant's serum, and the presence of such opsonins for certain strains in the serum of adults. On this account, we would prefer the term "acquired opsonins," although to avoid confusion we have retained the more commonly used term "natural opsonins" in this section.

In Table VII, we have recorded the phagocytic effect of natural opsonins on certain F and M variants. This experiment shows that opsonins are absent in infant's serum and present in varying amount for certain strains in the serum of adults.

Inasmuch as polyvalent antisera do not contain opsonins for virulent streptococci, we have titrated the natural opsonin in the serum of one adult in order to determine whether it was sufficiently concentrated to use in immunotransfusion. This titration is recorded in Table VIII. In theory, one might expect to benefit a child by transfusing him with 500 cc. of whole blood, containing natural specific opsonin.

DISCUSSION

As far as is definitely known at the present time, the body can destroy Gram-positive organisms in only one way—by their phagocytosis and intracellular digestion. In the first paper, we presented evidence showing that the virulence of the hemolytic streptococcus was correlated with the resistance of the organism to phagocytosis. In this paper, we have presented evidence for the truth of the corollary; *i.e.*, that immunity is correlated with the presence in the animal of specific opsonizing antibody against the virulent variants.

Although Day (12) has recently questioned the type specificity of streptococcus immunity, our own experiments and those of other workers in this field indicate that immunity is strictly type-specific. In view of the accumulating evidence that it is possible to achieve a low level of non-specific immunity to infection with the pneumococcus, we regret that we did not have the opportunity of repeating Dr. Day's experiments with the cultures which he so kindly sent us. In the present state of our knowledge, however, we feel that it is wiser to emphasize the importance of type specificity in streptococcal immunity.

The determination of the types of streptococci has been attempted by three methods: agglutination, precipitation, and mouse protection tests. We do not believe that any of these methods is suitable for the rapid typing of freshly isolated strains. The agglutinative reaction has been shown by Andrewes (5) and Williams (13) to depend upon a multiplicity of antigens and antibodies, necessitating the absorption of antisera. We have presented evidence, moreover, that the agglutination of old cultures of virulent streptococci does not necessarily identify the all-important opsinogen of the young, capsulated organism. The capsulated streptococci in young cultures apparently resist agglutination. It appears to us that the protective mechanism is dependent upon the presence of a specific opsonin capable of combining with the capsular material surrounding the young, virulent streptococcus, and is independent of those antibodies which combine with the somatic antigens and which may be demonstrated by agglutination of older cultures.

The precipitation technique of Lancefield (7) is accurate, but, again, requires the preparation of bacterial extracts and the absorption of antisera before results can be obtained.

The mouse protection test has the disadvantage of requiring strains of high virulence for mice, and many freshly isolated human-virulent strains may require many passages through mice before they attain this degree of virulence.

In proposing the opsonic method for typing the human-virulent variants of the hemolytic streptococcus, we believe that it is more pertinent than the agglutination test and is simpler and more rapid than the precipitation and mouse protection tests. Perhaps the main reason for the desuetude into which the opsonic method has fallen in recent years is the difficulty of interpreting the results. When we carried out the test in the ordinary way with 18 hour broth cultures, we too were often unable to detect the action of a specific opsonin, since even infant's blood can phagocyte many cocci in a culture of this age and the addition of a specific opsonin does not make a really decisive difference in the amount of phagocytosis. When, however, the virulent streptococcus is grown for a short time only in 50 per cent horse serum neopeptone water, practically no phagocytosis takes place in infant's blood, and the addition of specific opsonin has now a decisive

effect, giving results easy to interpret, as will be seen by referring to Table III.

Our difficulties have not been with the opsonic method, but with the preparation of opsonizing antisera. Lancefield (7) has reported similar difficulties in preparing the type-specific precipitin, and Williams (13) states that the "proportion of rabbits yielding good protective antiserum is not great." All are agreed that living cultures must be used in the course of immunization. One might suppose that living cultures have to be used in preparing antisera, because any agent that kills the coccus alters the protein capsular substance (probably identical with Lancefield's M substance), so that it is no longer antigenic. And one could draw an analogy with the anthrax bacillus, for here again living cultures have to be used in immunizing animals, and Tomcsik and Szongott (14) state that the capsular material of the anthrax bacillus is protein in nature. But against such a theory must be set the fact that mice can be actively immunized against the M variant of the hemolytic streptococcus by injecting the heat-killed organisms (15).

In view of the difficulty experienced in preparing effective antisera against the hemolytic streptococcus in laboratory animals, it is not surprising that the polyvalent antibacterial sera available for the treatment of streptococcal infections have proved valueless. Both Hare (11) and ourselves have been unable to demonstrate opsonic antibodies in such sera. Fothergill and Lium (16) report the absence of bactericidal antibodies in these sera. It would seem logical to attempt to produce effective opsonizing antisera against the commoner types of hemolytic streptococci, although it is true we have no evidence that they would be therapeutically successful. Such evidence might be obtained in another way. We have shown that adults have specific opsonins against certain strains of streptococci, and it would be practical to select a donor having the specific opsonin for transfusion of patients with streptococcus septicemia.

These studies are neither as thorough nor as complete as we would wish, but the work had to be discontinued at this point and the papers are published in the hope that the data may be of some assistance to other workers in this field.

CONCLUSIONS

1. An antiserum which specifically protects mice against a virulent culture (M variant) of the hemolytic streptococcus contains specific opsonin. Phagocytosis of the organisms can be observed in the peritoneum of the protected mouse.

2. An antiserum prepared by injecting an animal with the living M variant specifically opsonizes both the F and the M variant of the strain.

3. Evidence is presented which indicates the probable identity of the specific opsonin and the anti-M precipitin of Lancefield (7). Agglutination appears to be dependent upon a different antibody.

4. It is possible to type the hemolytic streptococci by means of specific opsonins, and the opsonic method has certain advantages over agglutination, precipitation, and mouse protection tests. It is evident from what little has been done that there are many types.

5. The serum of infants contains no opsonin for the virulent hemolytic streptococci, but the serum of adults may contain specific opsonins for certain strains. Inasmuch as no opsonins were demonstrable in two polyvalent antibacterial sera examined, the possibilities of therapeutic transfusion are discussed.

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