ACCESSORY PLASMA FACTORS INVOLVED IN THE BACTERICIDAL TEST FOR TYPE-SPECIFIC ANTIBODY TO GROUP A STREPTOCOCCI

II. Human Plasma Cofactor(s) Enhancing Opsonization of Encapsulated Organisms*‡

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Human bloods have been shown to vary in the efficiency with which they opsonize Group A streptococci in the presence of constant amounts of antibody to homologous type M protein (1, 2). This variation has been found to be due to either qualitative or quantitative differences in the accessory plasma factors that enhance phagocytosis of antibody-coated organisms.

The hyaluronic acid capsule of the Group A streptococcus has been implicated as an important factor in its resistance to phagocytosis (3–6). Recently, Hirsch and Church (7) have postulated that a "capsule-neutralizing" thermolabile factor in human plasma is required for the efficient phagocytosis of encapsulated strains.

This study will present evidence to support the concept that the capsule of the Group A streptococcus imposes an opsonic requirement for a thermolabile plasma factor(s) which is present in varying amounts in human bloods and which appears to be independent of the complement system. On the assumption that this is a plasma cofactor which further enhances the rate of phagocytosis of encapsulated organisms in the presence of antibody to M protein and possibly complement components, it will be referred to hereafter as streptococcal "coopsonin."

Materials and Methods

Strains of Streptococci.—Strains of streptococci were selected for use in the bactericidal test on the basis of their capacity to resist phagocytosis and to grow rapidly in human bloods in the absence of homologous type antibody. Most of these strains were freshly isolated from

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throat cultures of patients at Children's Memorial Hospital, the Naval Medical Research Unit Number 4, and the Northwestern University Clinics. Some additional strains were supplied to us by Dr. Rebecca Lancefield, The Rockefeller Institute, New York; Dr. Elaine Updyke, the Communicable Disease Center, Chamblee, Georgia; Dr. Armine Wilson, the du Pont Institute, Wilmington; and Dr. W. Barry Wood, Johns Hopkins Medical School, Baltimore. The strains of pneumococci employed were supplied by Dr. Colin M. MacLeod, New York University, New York.

To maintain strains in a phase of maximal resistance to phagocytosis, it was convenient to select those which were mouse-virulent so that they could be passed through mice whenever stock cultures showed signs of dissociating. Capsules were demonstrated by the wet India ink method as described by Wilson (8). In addition, highly encapsulated variants were selected by their tendency to form very short chains in 3 hour cultures in 20 per cent serum-broth (9). Semiquantitative estimates of M protein content of streptococcal strains were made by preparing hot acid extracts (10) of the sediments of 40 ml of 18 hour Todd-Hewitt broth cultures which had been adjusted to a turbidity reading of 100 on a Klett-Summerson colorimeter. Serial dilutions of the M protein extracts in 0.85 per cent sodium chloride solutions were tested for precipitation against standard lots of homologous, absorbed, type-specific rabbit antiserum in capillary precipitin tubes. Strains considered rich in M protein yielded extracts which, under these conditions, produced visible M–anti-M precipitates at M-extract dilutions of at least 1:40.

Variant strains of Group A streptococci were selected according to the presence or absence of M protein and hyaluronic acid capsules. Thus, four groups of variants were studied: M-positive, capsule-positive; M-negative, capsule-positive; M-positive capsule negative, and M-negative, capsule negative. The first category is referred to hereafter as "virulent" strains and the last as "avirulent."

Studies of Phagocytosis by Bactericidal Tests.—The bactericidal test employed was identical with that described in detail in previous studies (2). Fresh, lightly heparinized human blood was mixed with varying dilutions of a culture of Group A streptococci and with homologous type antiserum. The survival of organisms was determined by colony counts of pour plates made from the mixtures after 3 hours of incubation in a roller tube apparatus at 37°C. To demonstrate a strongly positive bactericidal effect, phagocytosis had to be rapid and efficient, otherwise the population of extracellular streptococci increased faster than it could be reduced by blood phagocytes. The system was, therefore, very sensitive to factors influencing the *rate* of phagocytosis. In the absence of thermolabile plasma factors, no significant bactericidal effect was detected against virulent streptococci despite the presence of optimal amounts of homologous antiserum (2). The method was, therefore, very effective for detecting variations in accessory plasma factors involved in opsonization.

For convenience, the results of the bactericidal tests were expressed as an index based upon the relative growth of streptococci in blood containing optimal amounts of homologous antibody compared with control samples in which type-specific antibody was absent (2). For example, a bactericidal index of 100 indicated that growth of the inoculum was 100 times greater in the control tubes than in the tubes containing homologous type antibody. Human bloods were compared for relative opsonic strength by determining their bactericidal effect in the presence of an excess of homologous type antiserum. Strongly positive tests were those with indices of 500 or greater. An index of 25 was considered the lower limit of significant opsonic activity. An index of 1 indicated uninhibited growth of the inoculum.

In experiments involving opsonization of avirulent strains of Group A streptococci, all human bloods studied showed a marked phagocytic effect which was only slightly enhanced by supplementing the system with rabbit antiserum prepared against these strains. The bactericidal index in such cases was derived by comparing growth of avirulent strains in cell-free

plasma with that in whole blood of the same donor. A slight modification of the bactericidal test was also necessary in studies of phagocytosis of pneumococci. Because of the slower growth of these organisms compared with streptococci, the period of the test was extended from 3 to 6 hours and the inocula were increased at least tenfold.

In view of the large number of variables inherent in the bactericidal test, experiments were designed so that, as often as possible, only one variable was tested in the same experiment. For example, a variety of strains with different properties were tested in the same experiment with the blood of a single individual, and, conversely, a single strain was tested in another experiment with the bloods of various individuals.

Experiments involving various modifications of plasma factors were made as follows: Freshly drawn heparinized bloods were centrifuged at 4°C and the supernatant plasma was separated from the cells. The sedimented blood cells were washed twice in 10 volumes of cold Tyrode-gelatin buffer and were reconstituted with equal parts of aliquots of the original plasma modified according to the experimental plan. White blood cell counts were made on the reconstituted mixtures and adjusted, when necessary, to the concentration of the original whole blood sample. No attempt was made to separate red from white blood cells, and indeed, an effort was made to vary conditions as little as possible from that of whole blood. By this method, the washed blood cells resuspended in fresh plasma produced bactericidal tests which were consistently as strong as those of whole blood.

Direct Opsonophagocytic Tests.—In some experiments, the rate of phogocytosis was observed microscopically by conventional direct opsonophagocytic counts. At frequent intervals during the 3 hour period of incubation of the bactericidal test, a drop of the test mixture was removed, spread on a glass slide and stained with Wright's stain. The conditions were identical to those of the bactericidal test except that the concentration of organisms in the culture was increased by about 1000 times to insure adequate numbers of phagocytized streptococci to count. 100 polymorphonuclear leucocytes were counted on each of 2 slides prepared from the contents of the same test tube. Phagocytosis was expressed as the percentage of polymorphonuclear leucocytes counted that contained streptococci (phagocytic index).

Complement Components.—Complement was measured quantitatively as described by Kabat and Mayer (11). Complement components were assayed by the reagent titration method described by Wedgwood (12). Properdin was assayed by the zymosan method of Pillemer and associates (13).

Bentonite Absorption Tests.—Wyoming bentonite, U.S.P. was prepared as a suspension of uniform particle size according to the method of Bloch and Bunim (14). Stock suspensions containing approximately 0.5 mg of bentonite per ml of water were employed and the well drained sediment of 0.3 to 3.0 mg of bentonite particles was resuspended in 1 ml aliquots of fresh plasma for 20 minutes at 37°C. The particles were removed by centrifugation in the cold at 15,000 × *G* for 30 minutes. Washed blood cells were then resuspended in equal volumes of the supernatant, bentonite-absorbed plasma and bactericidal tests were made as described above.

Absorption of Plasma Opsonins with Strains of Group A Streptococci.—18 hour Todd-Hewitt broth cultures of the variant strains of Group A streptococci studied were centrifuged and the sedimented cells resuspended in fresh Todd-Hewitt broth and adjusted to a turbidity reading of 100 on a Klett-Summerson colorimeter. Colony counts made from such standardized cultures averaged approximately 2×10^8 per ml. Sedimented cells from volumes ranging from 1 to 3 ml of these cultures were washed twice in 5 ml volumes of 0.85 per cent sodium chloride, resedimented, drained and suspended in fresh plasma. After incubation at varying temperatures for 20 minutes with frequent agitation of the tubes, the plasma-bacterial suspensions were centrifuged at approximately 15,000 × *G* for 30 minutes at 0°C and the supernatant plasma pressed through a Millipore filter (0.65μ). The filtered plasma was then employed as

a suspending medium for equal volumes of washed blood cells. Bactericidal tests were made with these reconstituted bloods against virulent strains of Group A streptococci in the presence of homologous type, heat-inactivated rabbit antiserum, as described above.

RESULTS

Specificity of the Opsonic Deficiency of Some Human Plasmas for Virulent Group A Streptococci.—In most of the studies to be reported, the bloods of two individuals (GHS and CL) were selected. These were found to be most strikingly deficient in opsonic activity against virulent Group A streptococci (2).

TABLE I

Bactericidal Tests Made With Human Bloods of Varying Streptococcal Coopsonin Content

Donor	Organism	Bactericidal Index‡
GHS*	Type 3 virulent streptococci	51
CL*	" "	64
MR	" "	8,578
KK	" "	6,336
GHS*	Type 3 pneumococci	54,656
CL*	" "	26,400
MR	" "	34,304
KK	" "	29,504
GHS*	Avirulent streptococci (T2/44/19)	5,232
CL*	" "	1,246
MR	" "	800
KK	" "	17,472

* Deficient bloods.

‡ (See Methods) Bactericidal index is an expression for inhibition of growth of streptococci in presence of homologous antibody compared with growth in control bloods in the absence of antibody. An index of 25 to 100 = 1 +; 100 to 200 = 2+; 200 to 500 = 3+; and greater than 500 is 4+ inhibition of growth.

These bloods will be referred to hereafter as "deficient" in contrast to the more usual behavior of most human bloods, referred to hereafter as "normal" (MR and KK in Table I). The deficient bloods produced weak bactericidal tests consistently during several years of observation against all typable, encapsulated strains of Group A streptococci tested. This deficiency could not be demonstrated, however, in any other phagocytic system studied. Avirulent Group A streptococci, containing neither demonstrable M protein nor capsules, were phagocytized and destroyed with equal efficiency by normal and deficient bloods. Similarly, virulent Type III pneumococci were destroyed equally well in the presence of homologous type antiserum by both kinds of blood (Table I).

The rate of phagocytosis by normal and deficient bloods also was determined by timed experiments in which blood smears were made at regular intervals

during the incubation period of the bactericidal test and the degree of phagocytosis was measured by direct opsonophagocytic counts (see Methods). Phagocytosis of virulent Group A streptococci was slower in the deficient bloods than in the normal bloods. This difference in the behavior of the two types of bloods was not apparent, however, when timed phagocytic tests were made

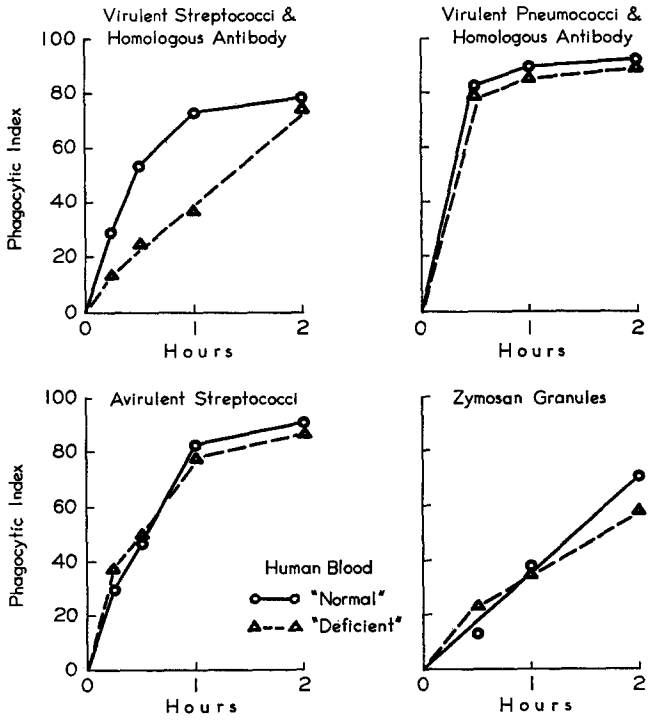

FIG. 1. Slow rate of phagocytosis of Group A streptococci by human bloods deficient in a coopsonin apparently not required for rapid phagocytosis of other agents studied. Phagocytic index refers to percentage of polymorphonuclear leucocytes counted which contain bacteria or particles (see Methods).

with avirulent Group A streptococci, virulent Type III pneumococci, or zymosan granules (Fig. 1).

The Relationship between Encapsulation of Group A Streptococci and the Phagocytic Requirement for a Plasma Coopsonin.—Variant strains of Group A streptococci were selected for bactericidal tests with normal and deficient bloods. The variants included encapsulated and non-encapsulated organisms. These were further subdivided into strains which did, and strains which did not, contain M protein.

In the absence of homologous type antibody, encapsulated strains rich in M

protein resisted phagocytosis and grew at optimal rates in all bloods studied. In the presence of homologous type antibody, all such strains were phagocytized efficiently by normal bloods (strongly positive bactericidal tests) and relatively inefficiently by deficient bloods (weakly positive bactericidal tests). Variants which possessed large capsules, but which did not contain M protein, were phagocytized to some extent by normal bloods, even in the absence of homologous type antibody. These strains were phagocytized much less efficiently by the deficient bloods, with or without the addition of homologous antiserum. Conversely, unencapsulated strains, with or without M protein in their cell walls, were phagocytized equally well by normal and deficient bloods, with and without the addition of homologous rabbit antiserum to the system (Table II). On the basis of these observations, it appeared that the presence of the streptococcal capsule imposed a requirement for a plasma opsonin which was not present to the same extent in all human bloods.

TABLE II

Relationship of Encapsulation of Streptococcal Strains to the Requirement for a Streptococcal Coopsonin in Human Plasma

Source of blood	Homologous antiserum	Bactericidal indices against streptococcal variants			
		Encapsulated		Non-encapsulated	
		M+	M−	M+	M−
Normal	None	1	18	19	358
Deficient	None	1	2	18	437
Normal	Present	768	51	1056	4074
Deficient	Present	83	3	912	3056

The Relative Requirement for Thermolabile Opsonin in the Phagocytosis of Encapsulated and Unencapsulated Streptococcal Strains.—The following experiments were made to determine the relative importance of thermolabile opsonins in the phagocytosis of various strains of Group A streptococci:

Washed blood cells were resuspended in equal volumes of fresh, normal human plasma which had been diluted progressively with the heated plasma (56°C for 30 minutes) of the same individual. Phagocytosis was studied by bactericidal tests made in the presence of a constant amount of antiserum to the homologous type of streptococcus.

A higher concentration of fresh plasma was required for optimal phagocytosis of encapsulated strains than that which was required to promote the phagocytosis of unencapsulated strains. For example, a 40 to 60 per cent dilution of fresh plasma in heated plasma resulted in almost complete loss of the bactericidal effect of the system against encapsulated strains, whereas comparable

dilutions of thermolabile factor(s) had less effect upon the rate of phagocytosis of unencapsulated strains (Table III).

Deficiency of Coopsonin in Human Bloods which Contained Normal Amounts of Complement.—The following experiments suggested that rapid phagocytosis of encapsulated streptococci required certain plasma opsonins, (other than complement), which were decreased or absent in some human bloods.

Fresh plasma from a normal individual, MR, was diluted in increasing amounts of heated plasma from the same individual, as in the preceding experiment. The fresh plasma of MR

TABLE III

Requirement for Thermolabile Plasma Factors in the Phagocytosis of Encapsulated and Non-Encapsulated Streptococci

Fresh plasma*	Bactericidal indices against streptococcal variants‡			
	Encapsulated		Non-encapsulated	
	M+	M−	M+	M−
per cent				
100	460	22	2624	3840
80	105	7	896	3328
60	55	6	768	704
40	7	1	371	51
20	1	1	352	26
0	1	1	16	13

* Fresh plasma diluted in the heated plasma of the same individual.

‡ Autologous washed blood cells were resuspended in plasma mixtures and bactericidal tests were made against streptococcal variants in the presence of inactivated homologous type rabbit antiserum.

was similarly diluted in the *fresh*, unheated plasma of GHS, a deficient donor. Washed blood cells of MR were then resuspended in these mixtures and bactericidal tests were made against highly encapsulated, M protein–rich strains of Group A streptococci in the presence of homologous type antiserum.

A representative experiment is shown in Table IV. Phagocytic activity decreased progressively when normal plasma was diluted in either heated, normal or in unheated, deficient plasma.

The Relationship of the Complement System to the Phagocytosis-Promoting Factor for Encapsulated Streptococci.—The opsonic deficiency in the fresh plasma of the human bloods studied could not be accounted for by a decrease in any of the 4 conventional components of complement, or of properdin. The sera of GHS and CL contained normal titers of C′1, C′2, C′3 and C′4, and contained between 6 and 10 units of properdin, measured by the zymosan assay. These deficient plasmas also contained normal virucidal titers against

Escherichia coli bacteriophage T2 r+, normal bactericidal activity against *Salmonella typhi*, *Shigella sonnei*,[1] and *E. coli*, and normal levels of lysozyme. Blood platelets did not appear to play any significant role in the opsonization of virulent streptococci. Bactericidal tests were no different with platelet-rich compared with platelet-poor preparations of plasma prepared from the bloods of either normal or deficient individuals.

Removal of any one of the individual components of the complement system from normal sera (preparation of complement reagents R1, R2, R3, and R4) resulted in marked decrease in the bactericidal tests against encapsulated

TABLE IV

Demonstration of Deficiency in Streptococcal Coopsonin in Human Plasma (GHS) Containing Normal Amounts of Complement

Plasma mixture		Bactericidal index T5*	Plasma mixture		Bactericidal Index T5*
MR fresh plasma	+ MR heated plasma		MR fresh plasma	+ GHS fresh plasma	
ml	*ml*		*ml*	*ml*	
1.0	—	2000	1.0	—	2000
0.8	0.2	2000	0.8	0.2	1904
0.6	0.4	134	0.6	0.4	525
0.4	0.6	33	0.4	0.6	105
—	1.0	1	—	1.0	42

* Washed blood cells of MR were resuspended in plasma mixtures and bactericidal tests were made against virulent Type 5 streptococci in the presence of homologous rabbit antiserum.

streptococci. It appeared, therefore, that complement either participated in the opsonization of these organisms or that the manipulations of the serum required to prepare these fractions destroyed some other very labile opsonin present in low titer.

Absorption of Streptococcal Coopsonin by Bentonite.—Because bentonite has been employed as an absorbent of a variety of serum factors (15–17) and does not, under certain conditions, absorb complement (18), it was employed in the following experiments in an attempt to selectively absorb streptococcal coopsonin.

Fresh plasma from normal bloods was absorbed with varying amounts of bentonite, ranging from 0.3 to 3.0 mg per ml of plasma, for 15 to 20 minutes at 37°C. The bentonite particles were removed by centrifugation at 15,000 × *G* for 30 minutes at 4°C. Washed blood cells were resuspended in equal volumes of bentonite-absorbed plasmas and the mixtures tested

[1] The bactericidal assays of deficient plasmas against *E. coli* bacteriophage, *S. typhi* and *Sh. sonnei* were kindly made by Dr. Ralph E. Wedgwood, Western Reserve University. Titers of complement components and of properdin also were confirmed by his laboratory.

for bactericidal activity against various strains of Group A streptococci in the presence of homologous rabbit antisera.

Absorption of plasma with bentonite resulted in marked decrease in coopsonin without loss of complement. The decrease in coopsonin was most apparent in bactericidal tests made with strains of Group A streptococci which possessed the largest capsules. Absorption of plasma with larger amounts of bentonite was required to reduce the opsonization of strains which possessed smaller capsules. Finally, opsonization of unencapsulated, M-negative strains was least affected by bentonite absorption of plasma (Table V).

The loss of opsonic activity of bentonite-absorbed plasmas was not associated

TABLE V

Effect of Absorption of Plasma With Bentonite Upon Cofactors Involved in the Opsonization of Virulent and Avirulent Group A Streptococci

Bentonite per ml plasma	C′	Activity of plasma in bactericidal test against:*	
		Virulent streptococci‡ (T3/34488)	Avirulent streptococci§ (T2/44/19)
mg	*μ/ml*		
0	38	563	1700
0.3	37	83	1134
0.6	38	13	340

* Washed blood cells were resuspended in the absorbed plasmas and bactericidal tests were made in the presence of heated homologous rabbit antiserum.

‡ Encapsulated, M protein–rich, mouse-virulent strain.

§ Non-encapsulated, M-negative, mouse-avirulent strain.

with significant decrease in total complement activity. Nevertheless, it was considered possible that there might have been a reduction in some component of complement that would not be detected by the hemolytic assay for whole complement. All four components of complement and properdin were assayed, therefore, before and after absorption of a normal plasma with bentonite (1 mg per ml of plasma). Bactericidal tests were then made against each of two encapsulated strains of M-rich streptococci by resuspending washed blood cells in the absorbed plasmas. A marked decrease in opsonic activity of the plasmas occurred following bentonite absorption in the absence of any significant change in the titers of the four components of complement, or of properdin (Table VI).

Attempts to Absorb Streptococcal Coopsonin on Highly Encapsulated Streptococcal Cells.—The possibility was considered that streptococcal coopsonin might combine directly with the hyaluronic acid capsule of Group A streptococci and might thus be absorbed selectively from plasma by highly encapsu-

lated strains. Accordingly, human sera, which were known to be free of antibody to the M protein of the homologous type organism, were absorbed with different variants of Group A streptococci and residual complement and coopsonin activity of the plasma were studied. When serum was absorbed at 37°C, complement activity and coopsonin activity were both markedly reduced by all strains except those which were very rich in M protein, and which possessed large capsules. The latter strains did not reduce components of complement significantly when suspended in a concentration of 2×10^8 to 6×10^8 organisms per ml of human serum at 37°C for 20 minutes. Furthermore, adsorption of homologous type M protein to these virulent strains prior to their suspension in

TABLE VI

Absorption of Streptococcal Coopsonin from Plasma by Bentonite

Absorbed with:	Complement titers (u/ml)*					Properdin	Bactericidal index	
	C′	C′1	C′2	C′3	C′4		T5‡	T6‡
						u/ml		
Unabsorbed	120	1280	480	960	1920	6	1280	320
Bentonite (1 mg/ml)	120	1920	320	640	1280	6	2	1

* Measurements of the components of complement are expressed in the units of the reagent titration technique of Wedgwood (12).

‡ Washed blood cells were resuspended in absorbed and unabsorbed plasmas, respectively, and bactericidal tests were made against virulent Type 5 and Type 6 streptococci, respectively, in the presence of homologous rabbit antiserum.

normal human serum, or absorption of human serum which contained homologous type M protein, did not reduce complement under the above conditions.

Absorption of serum at 37°C with these virulent strains of streptococci resulted in some decrease in opsonic activity but this result was not consistent, contrary to a preliminary report (1), and varied with individual human sera and with the strain employed. Avirulent organisms at this temperature removed both complement and opsonic activity. At 0°C, absorption of serum with avirulent strains in concentrations of 2 to 6×10^8 organisms per ml did not reduce complement but, again, reduction of opsonic activity was variable with individual sera and not marked. The addition of purified streptococcal hyaluronate in concentration of from 1 to 10 mg per ml did not interfere significantly with coopsonin activity. Higher concentrations of hyaluronate (up to 40 mg per ml) decreased phagocytosis but these concentrations were also anticomplementary. We were unable to demonstrate convincingly, therefore, a marked affinity between the streptococcal hyaluronate capsule and a serum opsonin.

Absorption of Coopsonin with Yeast Cell Walls (Zymosan).—It was considered

of interest to determine the effect of absorption of serum with cell walls of organisms other than streptococci to which most human sera contain either specific or cross-reacting "natural" antibodies. Sera were absorbed with zymosan at 17°C to prepare a "properdin-deficient" (RP) reagent. Some samples of RP were reabsorbed at 37°C to prepare a "properdin-free" (RPb) reagent, according to the method of Pillemer and associates (13). In addition, aliquots of the same individual's fresh serum were absorbed at 0° with the same amounts of zymosan (1.5 mg per ml of plasma) and with amounts up to 8 mg of zymosan per ml of plasma.

TABLE VII

Effect of Absorption With Zymosan Upon the Streptococcal Coopsonin Activity of Normal Human Plasma

Subject	Suspending medium for washed blood cells	Complement	Bactericidal indices against virulent strep
		u/ml	
LK	Whole plasma	33	2500
"	RP*	22	90
MH	Whole plasma	38	908
"	RPb‡	28	6
PB	Whole plasma	37	457
"	RPb	34	2
MR	Whole plasma	38	640
"	Absorbed at 0°C§	38	582

* RP, plasma absorbed at 17°C with zymosan (1.5 mg/ml).

‡ RPb, plasma absorbed at 17°C, and again at 37°C, with zymosan.

§ Absorption of plasma with amounts of zymosan up to 8 mg per ml at 0°C did not decrease bactericidal index.

Thrice washed blood cells were resuspended in aliquots of serum which had been absorbed at various temperatures with zymosan, and in unabsorbed serum from the same individual as a control. The reconstituted blood cell–plasma mixtures were then inoculated with cultures of virulent streptocci, homologous type rabbit antiserum was added, and bactericidal tests were made.

Absorption of serum with zymosan at 17°C resulted in marked loss of bactericidal activity of the reconstituted bloods (Table VII). The loss of activity was not associated with a comparable decrease in complement. The opsonin could not be removed at 0°C by zymosan in amounts up to 8 mg per ml of plasma. Under these conditions, complement was not reduced. The opsonic activity could not be restored by the addition of "purified properdin" (18) to system.[2] Streptococcal coopsonin was inactivated, however, by the addition to human plasma of a rabbit antiserum to human "purified properdin"[2] under

[2] Kindly supplied by Dr. Ralph Wedgwood.

conditions which did not reduce complement nor form visible antigen-antibody precipitates (19). From the above experiments, it appeared that the coopsonin for encapsulated streptococci could be absorbed or neutralized by antigen-antibody complexes other than those specific for streptococci and that it could be neutralized by antibodies made against the fraction of serum known to contain so called "properdin".

Sephedex Gel Filtration of Plasma Opsonins.—Because of the lability and small concentration of streptococcal coopsonin, dialysis and various plasma protein fractionation procedures which were attempted to further characterize this factor were found to be unsatisfactory. The molecular sieving effects of dextran gels (20) (Sephedex[3]) were found to be useful, however, in obtaining serum fractions of varying molecular size which could be tested for coopsonin activity.

Fresh, human serum was filtered through columns of Sephedex saturated with Tyrode's buffer. 3 to 5 ml samples of serum were passed through columns 15 cm high and 1 cm in diameter at 4°C. Under these conditions, effluent samples were obtained, within 30 to 40 minutes, which contained concentrations of serum protein and complement titers almost equal to those of the original, unfiltered serum. Equal volumes of washed blood cells were added to the effluent samples of gel-filtered serum proteins in Tyrode's buffer, and bactericidal tests were made against virulent streptococci.

Complete opsonic activity of the serum proteins was recovered after filtration through G 75 Sephedex in Tyrode's buffer. The molecular weight of streptococcal coopsonin appeared to be, therefore, greater than approximately 50,000. Gel filtration of the deficient plasmas through G 75 Sephedex did not increase their opsonic activity. The weak opsonic activity of these plasmas, therefore, could not be interpreted as being due to a plasma inhibitor with molecular weight of less than 50,000.

DISCUSSION

The resistance of Group A streptococci to phagocytosis by blood leucocytes has been shown to be related to the presence of at least two surface components, M protein and hyaluronic acid capsules (3–7). In the absence of these components, Group A organisms are opsonized by the bloods of all mammalian species studied. It is probable, therefore, that the M protein–rich, encapsulated Group A streptococcus owes its striking resistance to phagocytosis to the elaboration of a surface containing a highly specific, non–cross-reacting antigen (M protein) and a non-antigenic polysaccharide chemically identical with that of mammalian host tissues (hyaluronic acid). In the absence of antibody to M protein, such a surface might not be readily recognized as "foreign" by blood phagocytes. Previous studies by Wiley and Wilson (21) have shown that re-

[3] Pharmacia, Rochester, Minnesota

sistance to phagocytosis remains unchanged in streptococci killed by methods which produce minimal denaturation or alteration of cell surface, and that such resistance, therefore, does not appear to be due to the elaboration by living organisms of any active substance "toxic" to the blood phagocytes.

In support of the concept of relative "non-reactivity" of the surface of virulent streptococci with non–type-specific opsonic antibodies in plasma is the failure of such organisms to absorb complement from human sera in contrast to the marked complement-absorbing properties of avirulent Group A streptococci at 37°C. Furthermore, in the presence of homologous M antibody, absorption of complement by virulent organisms is not significantly enhanced. It would appear, therefore, that M–anti-M complexes in the hyaluronic acid gel matrix of the streptococcal capsule do not have marked affinity for complement.

The studies reported here develop further the line of investigation which, for several years, has pointed to an opsonic factor in human serum which results in efficient *in vitro* opsonization of virulent Group A streptococci. Rothbard's studies first demonstrated the increased opsonic power of human sera, compared with that of other mammals (5). Maxted (22) and Fleck (23) confirmed these differences and the former showed that monkeys also possessed the plasma opsonin effective against virulent streptococci. Stollerman, Kantor, and Gordon (2) showed individual variation in the quantity of opsonic accessory plasma factors within human and rabbit species. Subsequently, Hirsch and Church (7) demonstrated the critical role played by the hyaluronic acid capsule of Group A streptococci in determining a requirement for particular plasma opsonin(s). The studies presented here further demonstrate that the variations of opsonic power of human plasmas are due to relative deficiencies of the accessory plasma factor(s) required to opsonize encapsulated streptococci. In addition, they show the separate identity of this factor from conventionally defined components of the hemolytic complement system.

The relationship of the streptococcal coopsonin to the complement system is not entirely clarified by the studies reported. Although the accessory factor does not appear to be a complement component, it cannot be stated as yet that this opsonin operates in the absence of complement. Manipulations of serum which remove or diminish any component of complement markedly affects opsonization, either because the integrity of the complement system is required for full opsonic activity of the plasma, or because such manipulations also remove or destroy the coopsonin which appears to be present in very low titer. So far, the coopsonin has not been purified. Until this is accomplished it does not seem possible to determine whether or not coopsonin can act alone with the M–anti-M complex to enhance phagocytosis or whether it is a cofactor which is required to activate the complement system.

With regard to the latter hypothesis, there are several analogies. Recent studies have demonstrated, for example, 11S globulins in human sera which

enhance the hemolysis of sensitized red blood cells, or the precipitation of aggregated gamma globulin, and which appear to be separate from, and accessory to, the complement system (24–26).

An alternative explanation of the streptococcal coopsonin could be that it is a plasma factor which combines directly with hyaluronic acid and thus neutralizes, in some as yet unexplained way, the latter's anti-phagocytic effect (7). Such an hypothesis would be more attractive if it were possible to specifically absorb the coopsonin with encapsulated streptococci or if the coopsonin were readily neutralized by the addition to plasma of streptococcal hyaluronic acid. Neither was accomplished convincingly in this study.

The presence of a capsule and M protein on the cell surface, therefore, may impose a very critical requirement for a maximal concentration of the components of a more general phagocytosis promoting system, such as that described by Tullis and Surgenor (27). The latter authors demonstrated reduction of the opsonic activity of plasma for starch granules by absorption of serum with zymosan or with powdered barium sulphate. They, too, were unable to reactivate phagocytosis by the addition of purified properdin, and they considered their system to be independent of complement.

Opsonization of avirulent, non-encapsulated organisms may require a very small amount of such a factor(s), perhaps because of the more reactive nature of the antigen-antibody complexes formed with cell wall antigens other than M protein. The "deficient" bloods described in this study may have adequate amounts of phagocytosis promoting factors for such reactive complexes and the deficiency may not become apparent until formation of these antigen-antibody complexes are blocked or "masked" by an excess of M protein and hyaluronic acid at the cell surface. Under the latter conditions, M–anti-M complexes might require much higher concentrations of the same cofactors to stimulate phagocytosis. Some support for the latter hypothesis is provided by the fact that absorption of sera with sufficient amounts of bentonite eventually decreases opsonization of avirulent, as well as of encapsulated, streptococci without decreasing complement titers. This reduction in opsonic activity occurs even in the presence of excess amounts of heated antiserum to the homologous strain of organisms. Thus, bentonite appears to remove opsonins other than complement and specific antibodies which are involved in the phagocytosis of avirulent, as well as of encapsulated, streptococci. The affinity of bentonite for alpha and beta lipoproteins (17) is well known, and absorption of plasma with this substance may remove a group of plasma factors which seem to be involved in the acceleration of phagocytosis.

The biological or clinical significance, if any, of the coopsonin is not yet apparent. Preliminary studies of adults with advanced rheumatic heart disease do not reveal, so far, any consistent pattern of coopsonin deficiency compared with matched normal controls. Approximately 85 per cent of 100 individuals

studied have normal plasma coopsonin activity. The remainder show varying degrees of deficiency (28). Studies of possible variations of coopsonin activity during the acute phase of febrile reactions in normal donors are in progress. In one experiment, fever induced artificially with typhoid vaccine did not increase streptococcal coopsonin in the blood of one of the deficient donors (29).

SUMMARY

A study was made of the nature of the thermolabile plasma factors in human blood which promote the phagocytosis of Group A streptococci *in vitro* in the presence of optimal amounts of type-specific M antibody. The plasmas of individuals with strong opsonic activity (normal) were compared with those of some individuals whose opsonic activity was consistently weak (deficient).

A general relationship was established between encapsulation of streptococci and the opsonic requirement for thermolabile plasma factor(s). Marked differences in phagocytosis of Group A organisms by human bloods were demonstrated with encapsulated strains only. Human bloods deficient in the cofactor required for opsonization of encapsulated streptococci (coopsonin) showed a normal rate of phagocytosis against all other organisms and particles studied. Furthermore, coopsonin-deficient bloods contained normal levels of four components of complement, of properdin, of lysozyme, and of direct bactericidal activity against several species of Gram-negative organisms and of *E. coli* bacteriophage.

The independence of the streptococcal coopsonin from complement was also demonstrated by absorption of plasma with bentonite and with zymosan. Under appropriate conditions, the coopsonin was reduced without significant loss of complement.

The data support the concept that the capsule of the streptococcus imposes an opsonic requirement for a plasma factor(s) which is present in varying amounts in human bloods and which appears to be independent of the complement system. The possibility that it is accessory to the latter components has not been excluded.

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