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

I. Atypical Behavior of Some Human and Rabbit Bloods

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Group A streptococci are rapidly phagocytized and destroyed when they are grown in fresh human blood that contains antibody to M protein of homologous serological type. This observation is the basis of the so called bactericidal test which has been employed, with various modifications, to demonstrate type-specific streptococcal antibodies (1–7).

Rothbard demonstrated that complement and other thermostable plasma factor(s) are required in the opsonizing system of the bactericidal test (3 (*a*), (*b*), (*c*)). Under the conditions of the system, bloods of rabbits and other common laboratory animals are only weakly bactericidal and are unsuitable for use in this test. Additional recent studies by Fleck (5) and by Maxted (6) support the evidence that mouse and rabbit bloods are not efficiently bactericidal in the presence of anti-M antibody, but indicate that monkey blood behaves similarly to human blood and appears to contain all of the factors required for rapid phagocytosis and destruction of streptococci in vitro.

During the past several years, the authors have encountered exceptions to this general experience, both with rabbit and with human bloods (8). An occasional rabbit has been found whose blood is as effective as that of most human beings in the bactericidal system. Conversely, two apparently healthy human beings have been studied whose bloods are relatively ineffective in destroying virulent streptococci rapidly in the presence of homologous type antibody. This report demonstrates such variations and attempts to analyze some of the factors that may be involved in the mechanism by which virulent Group A streptococci are phagocytized and destroyed by blood leukocytes in

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the presence of type-specific antibody. It will be shown that the human bloods which are ineffective in the bactericidal test have some plasma deficiency which results in a slower rate of phagocytosis. Throughout this discussion, the term "normal" blood refers to blood of individuals that does not contain type-specific streptococcal antibody.

Materials and Methods

Strains of Streptococci.—Strains of Group A streptococci were selected for use in the bactericidal test on the basis of their capacity to resist phagocytosis and to grow rapidly in human and animal bloods in the absence of homologous antibody. It was convenient to employ strains that were mouse-virulent so that they could be passed through mice several times whenever stock cultures appeared to show signs of dissociating by becoming less resistant to phagocytosis.

The strains used in these studies included the D 24 strain of Type 30, the SF 42 strain of Type 12 and the S 43 strain of Type 6.[1] The rabbit virulent "Gay" strain (Type 30) was also employed to produce subcutaneous infections in rabbits.[2] Other strains of various serological types of streptococci were isolated from the throats of patients studied in the outpatient clinics of Northwestern University Medical School. All strains grew on rabbit blood agar with the formation of matt or mucoid colonies, and all produced strongly positive precipitin reactions when extracts of the organisms were tested against absorbed type-specific rabbit antisera[3] according to the method of Swift, Wilson, and Lancefield (9).

Fresh cultures (6 to 8 hours) of strains found suitable for use in the bactericidal test were mixed with equal parts of defibrinated rabbit blood and were frozen in dry ice–alcohol mixture. These mixtures were stored at −70°C. or were promptly lyophilized. Stock cultures were prepared by inoculating Todd-Hewitt broth with frozen or lyophilized cultures and incubating the tubes for 18 hours at 37°C. After one or two transfers in broth to stimulate active growth, stock cultures were kept in the refrigerator at 4°C. for 1 to 2 weeks. Overnight Todd-Hewitt broth cultures, made from these stocks, were used in all experiments.

Bactericidal Tests.—The method employed was almost identical with that recently described in detail by Lancefield (7). Sixteen to 18 hour Todd-Hewitt broth cultures were centrifuged at 3000 R.P.M. for 20 minutes and the sediments were resuspended in sufficient fresh media to produce a turbidity reading of 100 on the scale of a Klett-Summerson colorimeter. The standardized culture was then diluted 10^{-4} and further diluted serially 1:4, 1:16, 1:64, and 1:256 in Todd-Hewitt broth. The last 4 dilutions were used as inocula. Bacterial counts ranged from 200 to 500 bacteria in 0.1 ml. of the first culture dilution, to 5 to 15 organisms in 0.1 ml. of the last culture dilution.

Direct bactericidal tests were made by adding 0.1 ml. of each culture dilution to 0.3 ml. of fresh heparinized blood in each of a series of 4 tubes, 100 × 9 mm. Heparin[4] in excess of 10 units per ml. of blood was carefully avoided to prevent anticomplementary activity (3). The tubes were closed with sterile rubber stoppers and incubated promptly at 37°C. in an apparatus that rotated the tubes end over end at 8 R.P.M. to assure the constant mixing essential for adequate phagocytosis. After 3 hours, pour plates were made by inoculating 0.1 ml. of the

[1] Kindly supplied by Dr. Rebecca Lancefield.

[2] Kindly supplied by Dr. Beatrice Seegal.

[3] Some of the sera used were kindly supplied by Dr. Elaine Updyke, Communicable Disease Center, Chamblee, Georgia.

[4] Lederle's Heparin, 1000 U.S.P. units per ml. of solution, was found to be satisfactory. 0.1 ml. of this solution was added to 10 ml. of blood.

contents of each test tube into 5 per cent sheep or rabbit blood agar. The number of colonies were counted after overnight incubation at 37°C.

Indirect bactericidal tests were made by adding to each of four tubes 0.05 ml. of the serum or plasma to be tested for antibody. 0.1 ml. of each culture dilution and 0.3 ml. of normal heparinized blood were added to each tube and the same procedure as described for the direct test was followed with these test mixtures.

At the beginning of each test, pour plates were made from 0.1 ml. of the culture dilutions to determine the number of bacteria inoculated into each tube. This number was compared with the colony count of pour plates made after 3 hours' incubation of the test mixtures in the

TABLE I

Direct and Indirect Bactericidal Tests with Whole Blood and Serum of a Rabbit Immunized with T30 Streptococci

	Test mixtures		No. of colonies from culture dilutions*				Bactericidal index‡
			Inoculum				
	Serum	Whole blood	572	186	56	8	
			After 3 hrs. growth in test mixture				
Direct test		Immune rabbit 2-10	418	87	15	1	230
		§Normal " 6-5	∞	∞	C	1,596	
Indirect test	Immune rabbit 2-10	Normal rabbit 6-5	45	6	1	0	1,024
	Normal " 6-8	" " 6-5	∞	∞	C	696	

* Fourfold dilutions of a 10^{-4} dilution of a 16 to 18 hour culture (see Materials and 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 (see Materials and Methods). <25 = 0; 25–50 = ±; 50–100 = 1+; 100–200 = 2+; 200–500 = 3+; >500 = 4+ inhibition. ∞ indicates innumerable colonies with blood completely hemolyzed. C indicates confluent areas of hemolysis, usually 1500 to 2000 colonies.

§ Normal refers to blood or serum in which homologous antibody is absent.

rotator apparatus. A "positive control" containing homologous type rabbit antiserum of known high titer, and a "negative control" containing normal rabbit blood, were included with each series of tests.

A strain of streptococcus that resists phagocytosis and grows vigorously in normal blood is essential for a suitable test. An increase in the bacterial population of 64-fold or greater will occur during a 3 hour incubation period (inoculum × 2^6) when suitable strains are employed. Strains of low virulence that are poor in M protein do not resist phagocytosis by normal blood. Furthermore, the phagocytosis of strains poor in M protein is only slightly enhanced in the presence of antibody to M protein. Such strains are, therefore, not suitable for use in this test (7).

The results of direct and indirect bactericidal tests made with whole blood, and with serum, of a rabbit immunized against Type 30 streptococci is demonstrated by a typical protocol shown in Table I.

The Bactericidal Index.—For convenience in comparing the results of the bactericidal tests, a formula was derived to express the inhibition of growth of streptococci by type-specific antibody as follows:—

$$(1)\ \text{Bactericidal Index} = \frac{\text{Inhibition of growth in the presence of type-specific antibody}}{\text{Inhibition of growth in normal blood}}$$

The inhibition of growth of streptococci in both the numerator and the denominator may be expressed as the total number of bacteria inoculated into all four tubes at the beginning of the test divided by the total number of bacteria in all four tubes at the end of the 3 hour incubation period, as determined by colony counts from pour plates.

$$(2)\ \text{Bactericidal Index} = \frac{\dfrac{\text{No. organisms inoculated}}{\text{No. organisms after 3 hrs.' incubation in test serum}}}{\dfrac{\text{No. organisms inoculated}}{\text{No. organisms after 3 hrs.' incubation in normal serum}}}$$

For a suitable test, the denominator (the growth in the control tubes without antibody) should show an increase of the inoculum by a factor of 64 according to the formula for bacterial growth: Inoculum $\times 2^n$, where $n = 6$ generations. The denominator of expression (2) is usually approximately $\frac{1}{64}$. When the growth of the control is greater or less than 64 times, the nearest multiple of 64 is employed as the factor. The test is not considered satisfactory if the factor of growth in the control is less than 32 times the inoculum. Therefore, the numerator is calculated and multiplied by the appropriate factor (32, 64, or 128, etc.) to obtain the index. For example, the bactericidal index may be derived from the indirect test demonstrated in Table I as follows:—

Total inoculum = 572 + 186 + 56 + 8 = 822

After 3 hours in test = 45 + 6 + 1 + 0 = 52

Increase in population in the control (normal serum) = 696/8 = 87; nearest value of 2^n = 64

Therefore, bactericidal index = $822/52 \times 64 = 1{,}024$.

Thus, an index of 1 means that the growth in the test and control tubes are the same. An index of 100 means that the growth in the control tubes is 100 times greater than that in the test. Indices less than 25 were not considered positive for type-specific antibody; 25 to 50 was considered weakly positive; 50 to 200 positive; 200 to 500 strongly positive; and greater than 500 very strongly positive. This index was useful for comparing the relative antibody content of a group of antisera that were all tested under identical conditions in the same experiment. It is not, however, an exactly reproducible quantitative expression for type-specific antibody content since it may vary somewhat from day to day depending upon the conditions of the test, the strains employed, the source of normal blood, etc.

Antisera.—Rabbits were immunized by repeated intravenous injections of whole heat-killed streptococci according to methods previously described (10). Animals were bled before, at weekly intervals during, and for several weeks after each 6 week course of intravenous injections. At each bleeding direct bactericidal tests for antibody to M protein were made with fresh heparinized whole blood. Serum was separated from an aliquot of the same blood sample and antibody was also assayed by indirect bactericidal tests. Sera were separated under sterile conditions and were stored at 4°C. Preservatives were omitted for short term storage but merthiolate in concentration of 1 part in 10,000 could be added to serum without interfering with the test when prolonged storage of the serum was necessary.[5] Freezing and thawing

[5] The use of preservatives in serum should be avoided if possible, however, because they interfere with the growth of streptococci in another useful test for type-specific antibody recently described, the so called "long chain reaction" (11).

of sera was avoided because in occasional instances such a procedure resulted in loss of type-specific bactericidal activity for reasons not clearly understood. Also, such treatment of sera often produced cloudiness that interfered with capillary precipitation tests for M antibody.

In some early experiments, rabbits were immunized by subcutaneous inoculation of live cultures of streptococci, particularly when the rabbit-virulent "Gay" strain was employed. Inoculation of animals with live cultures, including the rabbit virulent strain, did not result in any significant difference in the development of type-specific bactericidal activity of the whole blood of these animals compared with that of animals immunized with heat-killed vaccines.

Strains of rabbits employed included New Zealand Red, Hare Brown, and Flemish Cross (a cross of Flemish bucks with New Zealand does, or with does of any long eared breed). Differences in bactericidal activities of rabbit bloods did not follow any particular pattern related to breed. Variations were found in all breeds tested.

EXPERIMENTAL

Variation in the Capacity of Rabbit Bloods to Destroy Streptococci in the Presence of Antibody of Homologous Type.—Various methods were used to immunize rabbits against Group A streptococci in order to study the development of type-specific antibody. The indirect bactericidal test was employed to detect antibody to M protein in the sera of the rabbits immunized. The rabbit sera were tested by adding them to "normal," heparinized human bloods that were known *not* to contain anti-M antibody to the homologous type organism employed in the test.

The whole blood of immunized rabbits was not considered suitable for direct bactericidal tests in view of studies by others which indicated that rabbit leukocytes are relatively ineffective in this system. To confirm these observations, direct tests were made with the whole blood of the immunized rabbits. It was an unexpected observation that direct tests made with the whole blood of some of the immunized rabbits showed relatively strong positive type specific bactericidal tests. Furthermore, two normal rabbits were encountered whose bloods became strongly bactericidal upon the addition of a rabbit antiserum that contained high titers of type-specific antibody. A representative experiment is shown in Table I. A positive direct bactericidal test is demonstrated with the whole blood of a rabbit (R 2-10) that was immunized with Type 30 streptococci. An even stronger indirect bactericidal test resulted when the serum of this rabbit was added to the whole blood of a normal rabbit (R 6-5). The bloods of both animals produced positive bactericidal tests against all serologic types of streptococci tested upon the addition of homologous type rabbit antisera. A comparison of the bactericidal activity of the blood of rabbit 6-5 and that of a "normal" human blood tested concomitantly in the presence of the same rabbit antiserum, is shown in Table II. The bactericidal effect is approximately equal in the two tests.

Further studies indicated that the blood of rabbit 6-5 was quite exceptional. As reported by others, the bloods of most normal rabbits tested were indeed relatively weakly bactericidal in the presence of type-specific antibody. Indi-

vidual variation from animal to animal occurred, however, and gradations from weak to strong tests could be demonstrated consistently with the bloods of selected animals. The relative bactericidal activity of the blood of five rabbits studied was consistent from day to day in repeated experiments made with the same strain of streptococcus and with different strains of other serologic types in the presence of homologous antibody.

Of 35 normal rabbits studied, the blood of only two produced indirect bactericidal tests that were as strong as those of the average normal human blood.

It was noted, however, that some rabbit antisera which produced strongly positive bactericidal tests with human blood were incompatible with the blood of rabbit 6-5, a blood that usually produced the strongest tests of all rabbits

TABLE II

*Comparison of Indirect Bactericidal Tests Made with Human and with Rabbit Bloods, T30 Streptococci, and Homologous Rabbit Antiserum**

Test mixtures		No. of colonies from culture dilutions				Bactericidal index
		Inoculum				
Rabbit serum	Whole blood	546	182	38	18	
		After 3 hrs. growth in test mixture				
Anti-T30	Normal human	47	11	12	0	704
Normal	" "	∞	∞	C	1,464	
Anti-T30	Rabbit 6-5	76	11	1	1	576
Normal	" "	∞	∞	1,648	526	

* See Table I footnotes for explanation of symbols.

studied. This is demonstrated in Table III. Indirect bactericidal tests were made with antisera to Type 6 streptococci obtained from four different immunized rabbits which were added to normal human blood. All four antisera produced strong bactericidal tests. When the same antisera were tested with the blood of rabbit 6-5, one of them (R 1-20 antiserum) failed to produce a strongly positive test. Another antiserum (R 1-21) produced a relatively weak test. This incompatibility was demonstrated consistently. No precipitin or complement fixation reactions, which may have accounted for the interference, could be demonstrated by mixing the serum of rabbit 1-20 with that of rabbit 6-5. The red cells of one animal were not agglutinated by the serum of the other. The cause of this incompatibility remains unexplained.

Bactericidal Tests Made with Type-Specific Antibody of Human Origin and Normal Rabbit Bloods.—The addition of human antiserum to normal rabbit blood resulted in bactericidal tests that varied with the particular rabbit blood employed; normal rabbit bloods that were weakly bactericidal in the presence

of rabbit antiserum behaved similarly in the presence of human antiserum. The bactericidal effect produced by a strong antiserum of either human or rabbit origin was determined by the innate capacity of the particular normal rabbit blood employed to produce a bactericidal effect.

Several different human antisera known to contain high titers of antibody to Type 12 M protein produced positive bactericidal tests when added to the

TABLE III

*Comparison of Indirect Bactericidal Tests with Human and with Rabbit Bloods Using T6 Streptococci and Homologous Rabbit Antisera Obtained from Different Immunized Animals**

Test mixtures		No. of colonies from T6 culture dilutions				Bactericidal index
		Inoculum				
Rabbit serum	Whole blood	528	112	28	12	
		After 3 hrs. growth in test mixture				
R 1-20—Anti-6	Normal human	20	0	0	0	2,309
R 1-21—Anti-6	" "	17	0	1	0	2,432
R 1-22—Anti-6	" "	16	4	0	0	2,309
R 1-23—Anti-6	" "	18	5	2	0	1,836
Normal rabbit	" "	∞	∞	C	528	1
		Inoculum				
		804	198	69	11	
		After 3 hrs. growth in test mixture				
R 1-20—Anti-6	Rabbit 6-5	C	504	220	80	25
R 1-21—Anti-6	" "	384	56	9	2	154
R 1-22—Anti-6	" "	50	13	5	0	1,024
R 1-23—Anti-6	" "	35	13	10	0	1,216
Normal rabbit	" "	∞	∞	C	1,092	1

* See Table I for explanation of symbols.

normal blood of rabbit 6-5. In addition, pooled human gamma globulin[6] which was known to contain Type 12 antibody produced a positive bactericidal test when added to a suitable normal rabbit blood. From these results it appears that human type-specific antibody can be measured adequately when added to rabbit blood from a suitably chosen animal. Unfortunately, such animals appear to be rare.

Variation in the Capacity of Human Bloods to Destroy Streptococci in the Presence of Antibody of Homologous Type.—Previous reports indicate that normal human blood destroys streptococci efficiently in the presence of adequate

[6] Kindly supplied by Hyland Laboratories, Los Angeles.

amounts of antibody to homologous M protein under the conditions of the bactericidal test. It was, therefore, an unexpected finding that the blood of some presumably normal individuals failed to do so. Of 25 healthy individuals tested, two were found to have blood that was relatively ineffective in the bac-

TABLE IV

Comparison of Human Bloods That Produce Strong Bactericidal Tests (RK, LF, PB, WK) with Bloods of Two Individuals (CL and GHS) That Produce Weak Bactericidal Tests

Human bloods	Date of test	Lot No. of antiserum Anti-12	Strain of culture Type 12	Bactericidal index
LF	Feb. 19, 1957	R1200 C	SF 42	2,000
GHS	" " "	"	"	148
PB	Mar. 8, 1957	"	"	6,600
GHS	" " "	"	"	27
RK	May 28, 1957	R1200 B	"	3,400
CL	" 21, "	"	"	11
RK	Oct. 1, 1957	"	"	9,660
CL	" 2, "	"	"	1
		Anti-6	Type 6	
PB	Jan. 30, 1957	Pool R9	S43	2,020
GHS	" " "	"	"	125
LF	Oct. 15, 1957	R603 A/2	"	9,750
CL	" 2, "	"	"	3
		Anti-30	Type 30	
LF	Mar. 19, 1957	A210	D24	8,340
GHS	" " "	"	"	35
		Anti-"Red Lake"	"Red Lake"	
CL	Sept. 18, 1957	LMRL	RLI	5
WK	Oct. 3, "	"	"	178

tericidal test against all strains of streptococci tested and with several antisera of both rabbit and human origin. The inefficient bactericidal activity of the blood of these two individuals was consistent when they were tested repeatedly during periods of 16 and 7 months, respectively (Table IV).

The differences in bactericidal efficiency of the human and rabbit bloods studied were shown to be quite reproducible when tests were carried out under identical conditions on the same day, employing the same antisera and the same

cultures in all experiments (Table V). Rabbits 6-9, 6-4 and 6-7 demonstrated the characteristic weak bactericidal test (low bactericidal index) produced by most normal rabbit bloods. The blood of rabbit 6-8 was relatively strong but not quite as good as most human bloods. The blood of rabbit 6-5 was as active as most human bloods. Of the human bloods tested in the same experiment, those of GHS and CL represent the exceptional very weakly bactericidal human blood; the blood of JO is active but usually less so than that of PB or LK. The latter two bloods represent the characteristic strong bactericidal tests that are observed with most human bloods in the presence of antibody to M protein.

Bactericidal Activity of Washed Leukocytes Resuspended in Plasma of Different Individuals.—To determine whether the weak bactericidal activity of the atypical human bloods studied was due to a cellular, or to a plasma defect, the following experiments were performed:

TABLE V

The Relative Bactericidal Activity of Rabbit and Human "Normal" Bloods in the Presence of Rabbit Antisera to Type 30 and to Type 6 Streptococci

Bactericidal indices* of rabbit bloods			Bactericidal indices* of human bloods		
Rabbit bloods	Antiserum		Human bloods	Antiserum	
	T30	T6		T30	T6
Rabbit-6-9	25	33	GHS	10	22
" 6-4	44	40	CL	25	14
" 6-7	81	52	JO	325	1,887
" 6-8	314	275	PB	423	2,410
" 6-5	1,340	1,055	LK	955	11,065

* See Methods and Materials for explanation of bactericidal index.

Freshly drawn heparinized bloods from different individuals 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 as described by Rothbard (3) and were reconstituted with equal parts of fresh heparinized plasma obtained from the same and from different individuals. White blood cell counts were made on the reconstituted mixtures and adjusted, when necessary, to approximately equal concentrations. The usual bactericidal tests were then made with the reconstituted mixtures, employing the same type-specific antiserum and streptococcal culture in each experiment.

The washed blood cells of GHS and CL became strongly bactericidal when resuspended in heparinized plasma of individuals whose whole blood was strongly bactericidal. Conversely, when the plasmas of GHS and of CL were used to resuspend the washed blood cells of individuals whose whole blood had strong bactericidal activity, the bactericidal tests were very weak (Table VI). It appeared, therefore, that the blood cells of GHS and CL behaved normally

in suitable plasma and that the defect in the activity of the whole blood of these individuals could be localized to their plasma. The results were consistent in repeated experiments made with three different serological types of streptococci and did not vary in experiments performed repeatedly over a period of several months.

Similiar experiments were attempted with rabbit bloods of varying bactericidal efficiency. Unfortunately, the two rabbits whose bloods showed the

TABLE VI

Indirect Bactericidal Tests Made with Washed Human Blood Cells Resuspended in Plasma from the Same, and from Different, Individuals

Washed blood cells	Human plasma	Rabbit serum	Bactericidal index
Bactericidal tests made with T30 cultures			
GHS	GHS	Anti-30	35
GHS	PB	"	380
GHS	LF	"	8,340
GHS	LK	"	475
GHS	GHS	Normal	1
LF	GHS	Anti-30	28
LF	PB	"	128
LF	BM	"	155
LF	LF	"	256
LF	LF	Normal	1
Bactericidal tests made with T19 cultures			
CL	CL	Anti-19	40
CL	RK	"	2,500
RK	CL	Anti-19	46
RK	RK	"	6,500
RK	RK	Normal	1

strongest bactericidal activity died before these experiments could be completed. The results obtained with the less active bloods of other rabbits were not conclusive. It is, therefore, not yet certain that variations in bactericidal activity of rabbit bloods are due to plasma factors alone. Attempts to find other rabbits whose bloods have bactericidal activity approaching that of the average human blood have failed so far. Attempts to resuspend washed rabbit cells in human plasma failed to result in strong bactericidal tests, confirming the observations of Rothbard (3).

Attempts to Measure Phagocytosis of Group A Streptococci by Bloods of Varying Bactericidal Efficiency.—Experiments were carried out to determine whether or not the variations in bactericidal activity of some human and rabbit bloods

would be reflected in demonstrable variation in the rates of phagocytosis of streptococci by leukocytes.

At frequent intervals during the 3 hour period of incubation, a drop of the test mixture of blood, antiserum and broth culture, was removed and spread on a glass slide for subsequent microscopic examination. The conditions were identical with those employed in the bactericidal test except that the concentration of organisms in the culture was increased by about 1000 times to insure adequate numbers of phagocytosed streptococci to count. Accordingly, standardized cultures (Klett reading of 100) were diluted 10^{-2} and 0.1 ml. of the dilution was inoculated into tubes containing 0.3 ml. of heparinized blood and 0.05 ml. of homologous antiserum. Control tubes containing 0.05 ml. of normal rabbit sera, instead of antiserum, were included with each experiment to determine the degree of phagocytosis that occurred in the absence of type-specific antibody. Indirect bactericidal tests were made simultaneously with the same strains of organisms and the same antisera in all experiments. Blood films were stained with Wright's stain. One hundred polymorphonuclear leukocytes were counted on each of two slides prepared from the contents of the same test tube. Phagocytosis was expressed as the percentage of polymorphonuclear leukocytes counted that contained streptococci (phagocytic index). In most experiments blood films were made at intervals of 15 and 30 minutes and at 1, 2, and 3 hours during incubation and rotation of the tubes.

The blood of GHS and of CL demonstrated consistently a lag in phagocytosis during the first 1 to 2 hours of the test when compared with other human bloods. During the latter half of the 3 hour incubation period, however, the leukocytes of GHS and of CL showed a marked increase in phagocytic activity so that at the end of 3 hours the percentage of leukocytes containing streptococci was usually almost as great as that observed with other human bloods tested (Fig. 1). The lag in phagocytosis was always most striking in the 1st hour of the incubation period. During this lag period the population of inoculated streptococcal cells could escape phagocytosis and multiply rapidly. This could result in a large population of viable extracellular streptococci at the end of the test.

The lag in phagocytosis that was observed with the bloods of GHS and of CL was shown to be due to plasma, rather than cellular factors. Timed phagocytosis experiments were carried out with washed leukocytes that were resuspended in the plasma of different individuals. The method of washing leukocytes was identical with that described above.

When the washed blood cells of GHS and of CL were resuspended in plasma of individuals whose bloods produced good bactericidal tests, there was no lag in phagocytosis and the time curve of phagocytic activity was identical with that observed when whole normal human bloods were studied (Fig. 2 *a*). Conversely, when the washed blood cells of normal individuals were resuspended in the plasma of GHS or of CL there was a marked lag in phagocytic activity (Fig. 2 *b*). It appeared, therefore, that the plasma factors of GHS and of CL that were responsible for weak bactericidal tests were also associated with a lag in phagocytic activity.

When human plasma was heated at 56° for 40 minutes to inactivate com-

plement (and properdin), and the heated plasma was employed to resuspend washed human blood cells, phagocytosis was greatly reduced throughout the 3 hour incubation period. There was no delayed rise in phagocytic activity in the latter part of the incubation period in contrast to the behavior of the unheated bloods of GHS and CL (Fig. 1).

Phagocytosis appeared much slower, in general, in rabbit bloods than in human bloods throughout the 3 hour incubation period (Fig. 1). Like the bloods

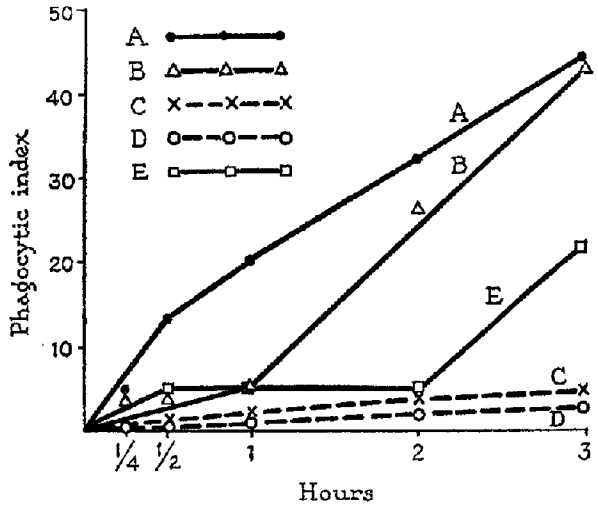

FIG. 1. Rates of phagocytosis of streptococci in blood from various sources to which type-specific antiserum was added. Each point represents the mean of 6 experiments.

A, typical normal human bloods.
B, atypical human bloods of GHS and CL.
C, washed normal human leukocytes resuspended in heated plasma.
D, control tests with typical normal human bloods to which normal rabbit serum, rather than antiserum, was added.
E, typical normal rabbit bloods.
See text for details.

of GHS and CL, the typical rabbit bloods that produced poor bactericidal tests, also showed a marked lag in phagocytic activity in the early part of the incubation period. Those rabbits whose blood produced good bactericidal tests (rabbits 6-5 and 2-10) died before timed phagocytic experiments could be made with their bloods. Phagocytic activity in the early stages of the incubation period, the time that is most critical to the outcome of the bactericidal test, unfortunately was not determined. The bloods of rabbits 6-5 and 2-10 were studied for phagocytic activity only at the end of the 3 hour incubation period in experiments made before the animals died. At the end of the 3 hour period

of incubation the blood of these animals did not show significantly greater phagocytic activity than that observed in the bloods of other rabbits.

Total white blood cell counts and differential counts made before and after the 3 hour incubation period of the blood antiserum-culture mixtures did not show a significant reduction in the numbers, or change in the appearance, of polymorphonuclear leukocytes in the bloods of GHS and CL compared with other human bloods. There was no indication, therefore, that destruction of polymorphonuclear leukocytes by streptococci could account for the inefficient bactericidal activity of these bloods.

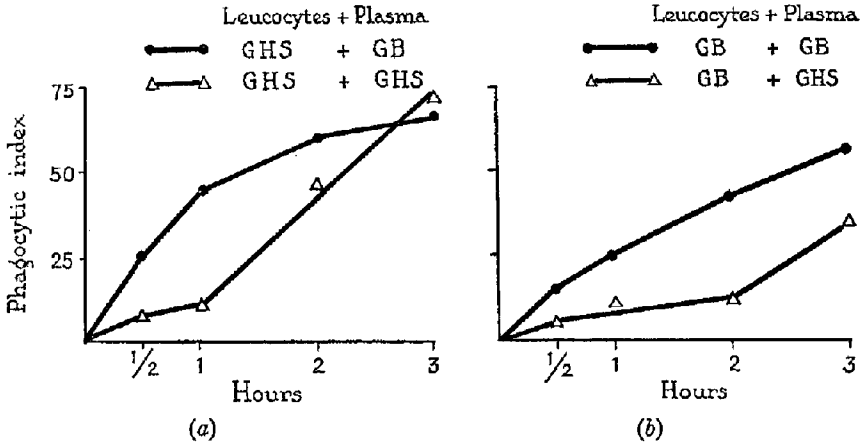

FIG. 2. Rates of phagocytosis of streptococci, in the presence of homologous type antibody, by washed human blood luekocytes resuspended in human plasmas. Fig. 2 *a* compares phagocytosis by the leukocytes of GHS resuspended in the plasma of GB (typical normal human), and in his own plasma. Fig. 2*b* compares phagocytosis by the leukocytes of GB resuspended in his own plasma, and in the atypical plasma of GHS.

DISCUSSION

The bactericidal test has proven to be of considerable value for studying the occurrence and specificity of M antigens in Group A streptococci and the corresponding anti-M antibodies in human and animal sera. It is inconvenient that no animal substitute for human blood has been satisfactory for use in the indirect test (with the exception of monkeys) especially when extensive testing by this method must be done. The finding of an occasional rabbit whose blood behaves as satisfactorily in the test as do most human bloods suggests that more extensive surveys of individual rabbits is warranted. If this characteristic is genetically determined, such rabbits might be bred for this purpose. Lancefield has recently reported one such animal in her laboratory (7). Aside from this practical consideration, the differences in the behavior of human and animal bloods in the bactericidal test has not been understood and raises many

questions about the mechanisms involved in the process by which Group A streptococci are phagocytized and destroyed in blood.

The bactericidal test depends upon a complex system that requires rapid phagocytosis and destruction of virulent Group A streptococci that have been opsonized by the binding of anti-M antibody to their surface in the presence of complement and other plasma factors that are not clearly defined. Many variables in the method have been emphasized (3, 7). Particularly important are the strains of streptococci employed. Those sufficiently rich in M protein resist phagocytosis and grow well in blood that is free of anti-M antibody. The same strains are destroyed rapidly by phagocytes in suitable bloods that contain this antibody. Bloods suitable for the production of strong bactericidal activity are those with the necessary plasma factors that speed phagocytosis and that contain blood leukocytes capable of ingesting and destroying the organisms.

Factors that are anticomplementary have a profound effect upon the test by slowing the rate of phagocytosis. Rapid phagocytosis is essential to demonstrate a positive bactericidal test. The organisms inoculated must be ingested and destroyed before the population of extracellular cocci increases at a rate that exceeds the capacity of the phagocytes to significantly reduce their numbers. The population of extracellular streptococci is capable of quadrupling within 40 minutes. For this reason the size of the inoculum is critical and the phagocytic events of the 1st hour probably determine the outcome of the test.

Plasma factors that influence phagocytic rates are, therefore, extremely important to the test. For example, if an excess of heparin is employed to prevent coagulation of the blood, its anticomplementary activity (and perhaps other effects) causes marked decrease in bactericidal activity (3). This decrease in bactericidal activity is associated with a striking diminution of phagocytic activity that can be demonstrated in blood films prepared from the test mixtures. Similarly, when washed blood cells are resuspended in plasma which has been heated sufficiently to destroy complement, and other thermolabile factors, such as properdin, the activity of the system is greatly diminished even in the presence of high concentrations of anti-M antibody (12). This, too, can be shown to be associated with a striking diminution of phagocytosis in blood film preparations (Fig. 1).

The observations reported here introduce still another variable into the bactericidal test, that of the nature of the "normal" blood employed. It can no longer be assumed that all human blood from apparently healthy individuals is suitable for use in the test. Denny (13) has also encountered an occasional individual whose blood was found unsuitable for use in bactericidal tests and he was unable to employ such bloods in the course of extensive studies he and his colleagues have reported on the measurement of human type-specific antibody (4).

The defect in human bloods described in this report appears to be relatively

rare. Neither of the two individuals in whom deficient bactericidal activity was detected have had any illnesses related to streptococcal infections or their sequelae. Neither are unduly susceptible to respiratory or other infections. Furthermore, the defect has been found to be constant during a period of more than 2 years. The possibility that the plasma defect is genetically transmitted is being studied.

It is clear that the defective bactericidal activity of these bloods resides in their plasmas rather than in their leukocytes. A subsequent report (12) will show that the plasma defect in these individuals does not appear to be a deficiency of complement or of properdin but rather involves additional plasma components of the opsonizing system that are as yet undefined.

Under the conditions of the experiments performed it seems clear that the plasma defect described results in a delay of phagocytosis in a manner that is too subtle to be detected if assays of phagocytic activity are made only at the end of the 3 hour test.

In the phagocytosis experiments described, the inoculum of streptococci was 1000 times greater than that usually employed in the bactericidal test. Under such conditions changes in the rate of phagocytosis may not be apparent to the same degree as when smaller inocula are employed. A delay in the activation of the plasma opsonizing system (complement and cofactors), perhaps the result of missing plasma factor(s), could have a marked effect on the bactericidal test and could explain the unusual behavior of the bloods of GHS and CL. An alternate possibility might be that certain plasma factors influence not only the rate of phagocytosis but the ultimate destruction of streptococci following their ingestion by leukocytes.

As in the case of experiments with human bloods of varying bactericidal activity, differences in phagocytosis were not demonstrated between weakly and strongly bactericidal rabbit bloods at the end of 3 hours. Whether differences in the early rate of phagocytosis occur in rabbits remains to be determined. Also, as will be indicated in a subsequent report, differences in the bactericidal activity of the rabbit bloods were not explained by variations in levels of serum complement or of properdin (12).

Several workers have commented that phagocytosis of streptococci by the leukocytes of animal bloods appears to be surprisingly active despite poor bactericidal activity (14). It has been considered that the poor bactericidal activity of animal bloods may be the result of the inability of the blood leukocytes of some species to destroy streptococci following ingestion. Recently Fleck (5) attempted to determine whether Group A streptococci survive ingestion by mouse leukocytes more often than they would survive ingestion by human leukocytes. Although his microdissection technique for isolation and cultivation of individual leukocytes containing ingested streptococci was ingenious, the number of experiments was considered to be too small to yield

significant differences in the behavior of the leukocytes of two species. Differences in the rates of phagocytosis of mouse and human leukocytes were not measured. In view of the observations reported here, slower phagocytosis may account for the poor bactericidal tests observed with various animal bloods.

Knowledge of the normal opsonizing system of human plasma is still very rudimentary (15) and the full significance of the activity of this system upon various leukocyte functions in blood is not known. Because of its sensitivity to the rate of phagocytosis the bactericidal test system employed in the present studies offers an opportunity for further investigation of these plasma factors.

SUMMARY

The bloods of two apparently healthy human beings, of 25 studied, failed to produce a strong bactericidal test for type-specific antibody to the M protein of group A streptococci under *in vitro* conditions wherein most human blood leukocytes rapidly phagocytize and destroy virulent organisms in the presence of anti-M antibody and accessory plasma factors.

The defect in bactericidal activity of these two individuals is associated with the plasma rather than with the blood leukocytes. Leukocytes suspended in these atypical plasmas showed a characteristic delay in the rate of activation of phagocytosis.

Although previously the bloods of laboratory animals (except monkeys) had been reported to be much less active than human blood in this system, occasional exceptions were encountered in rabbits in this study. Two rabbits were found whose bloods were as strongly bactericidal against streptococci, in the presence of type-specific antibody, as the blood of the average "normal" human being.

The atypical behavior of some human and rabbit bloods in the bactericidal test may be explained by variations in accessory plasma factors that are as yet unidentified and that influence the rate of phagocytosis of virulent streptococci *in vitro* in the presence of type-specific antibody.

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