BACTERIOSTATIC EFFECT OF HUMAN SERA ON GROUP A STREPTOCOCCI

II. Comparative Bacteriostatic Effect of Normal Whole Blood from Different Animal Species in the Presence of Human Convalescent Sera

By SIDNEY ROTHBARD, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

(Received for publication, April 24, 1945)

The demonstration by the indirect bacteriostatic test of type-specific antibodies in sera of patients convalescing from group A streptococcal infections required the observation of certain precautions: the prevention of culture dissociation by keeping the streptococci frozen; the use of young, actively growing cultures; and the utilization of afebrile children's blood which in itself did not inhibit streptococcal growth. Later age groups were carefully excluded as a source of blood for this test since, in this group, the blood was frequently bacteriostatic without the addition of convalescent serum (1). In view of the difficulty in obtaining blood suitable for the indirect test in quantities sufficient to test many sera, the question arose whether blood other than human could be used. Apparently neither immune animal serum nor convalescent human serum alone inhibits the growth of Gram-positive bacteria, but the active participation of leukocytes is required. Complement also may function to accelerate the sensitization of these bacteria by the antibodies, and thus permit more rapid phagocytosis by the leukocytes. Although Ward and Enders (2) found that complement was not required for phagocytosis of virulent pneumococci, provided the test was continued for 8 hours, they found that with complement the same results were obtained in 2 to 3 hours. At least two components of blood, leukocytes and complement, must therefore be considered as essential elements in the 3 hour test used in the present study; and the serum or plasma was shown in the experiments reported here to supply additional necessary factors.

The work of Dingle, Fothergill, and Chandler (3) is pertinent to the question whether blood of heterologous species may be used; they showed that complement of some heterologous animal species failed to activate the bactericidal action of immune sera for *H. influenzae*. These problems, however, have not been thoroughly investigated with reference to bacteriostasis of group A streptococci since the antigenic composition of these organisms has become better known. Consequently, it is uncertain whether the same principles hold for group A streptococci as for other microorganisms. Moreover, because clotting

of the blood used in a bacteriostatic test must be avoided, some form of anticoagulant is required; and its effect on bacteriostasis must be properly evaluated.

The effect on phagocytosis of different methods for preventing the blood coagulation has been investigated by numerous workers. Hamburger (4) and Colebrook and Storer (5) reported a harmful action of calcium-neutralizing agents on phagocytosis; Wising (6) demonstrated the complement-fixing properties of heparin salts; Ward (7) noted the reduction of leukocytes by defibrination of blood, and confirmed Fleming's (8) observation that this reduction alters the results of opsonization experiments. These reports indicate some objection to each of the commonly used anticoagulants. Thalhimer and Colwell (9) concluded from their studies, however, that defibrinated blood was more bactericidal than heparinized blood, although they felt the technique was best standardized by the use of heparin. Kuttner and Lenert (10) also found heparin satisfactory.

The present investigation has, therefore, been extended to determine the effect of these anticoagulants on the children's blood used in the following experiments, and to study certain other properties of blood of different species which may affect the bacteriostatic test under consideration.

Materials and Methods

The experimental procedures have been detailed in the previous report (1). The convalescent sera from the same 3 patients previously studied furnished the antibody: H. A., infected with group A, type 6 streptococci; E. B., infected with type 19; and C. DeM., infected with type 26. The antigens were strains of streptococci isolated from the nasopharynx of each of these patients.

Whole Blood.—The blood was usually supplied by a single child or occasionally by two and pooled when a larger amount was needed. Animal blood was obtained by cardiac puncture from rabbits of mixed stock weighing approximately 1800 gm., or from 350 gm. guinea pigs, or by venepuncture from sheep weighing 35 to 50 kilos. All blood was drawn not more than 1 hour before each experiment; pooling of blood was not necessary except, as noted, in the case of children.

Anticoagulants.—Human blood was defibrinated by shaking with glass beads in a sterile flask or else was prevented from clotting by mixing with potassium and ammonium oxalate to give a concentration of 0.12 and 0.08 per cent respectively, or by mixing with sodium citrate to make a final concentration of 0.95 per cent. Heparin (Connaught Laboratory, Toronto University) proved to be the most satisfactory anticoagulant; it was dissolved in physiological saline to make a 1:700 dilution and autoclaved for 30 minutes at 15 lbs. pressure. Heparin in a final dilution of 1:16,000 for human blood; 1:8,000, for rabbit or sheep; and 1:5,600, for guinea pig was sufficient to prevent coagulation.

Preparation of Plasma-Blood Cell Mixtures.—When the blood cells were separated from the plasma, the heparinized blood was centrifuged at 1,200 R.P.M. for 5 minutes, the plasma removed, and the corpuscles washed 3 times in 5 volumes of Locke's or Tyrode's solution with care to prevent injuring the leukocytes. The blood cells were promptly mixed with plasma, and the volume restored to that of the original whole blood.

EXPERIMENTAL RESULTS

Studies on Anticoagulants.—A comparative study of different anticoagulants was made under the conditions of the bacteriostatic test described previously (1). Whole blood from a child free of any infection was divided into 4 parts. Each portion was prevented from clotting by one of the following methods: defibrination, the addition of heparin, potassium and ammonium oxalate, or sodium citrate. The leukocyte counts on the last 3 specimens were approximately equal, but that on the defibrinated portion was about 3,000 cells per c. mm. lower than the others. A marked difference in bacteriostatic activity was observed when defibrination or the calcium-neutralizing agents, potassium and ammonium oxalate or sodium citrate, were employed, as compared with heparin.

To determine whether partial replacement of calcium ions would increase the degree of bacteriostasis, oxalated and citrated bloods were each divided into two 2 cc. portions: to one part of each, 0.05 cc. of 2 per cent calcium chloride was added and the other was used for a comparative control. Results, although not consistent, suggested that partial replacement of the calcium ion permitted more bacteriostasis to occur. Complete counteraction of the citrate or oxalate ions with calcium was not possible because the blood clotted. Another experiment comparing the effect of 0.45 and 0.95 per cent citrate concentrations showed that the lower concentration of citrate allowed greater bacteriostasis.

Because defibrinated blood with approximately one-half the number of leukocytes was less effective than heparinized blood (Table I), it seemed advisable to find out the critical range of leukocytes for optimal bacteriostatic activity. Another sample of heparinized human blood was centrifuged and the plasma was removed. The plasma and cells were then remixed in varying proportions to give a range between 6,600 and 1,200 leukocytes per c. mm.; and the mixtures were used in the bacteriostatic test summarized in Table II. Progressively decreased bacteriostasis roughly paralleled the diminution in leukocytes, and the critical level was between 4,300 and 3,000 leukocytes per c. mm. Although defibrination did not always reduce the number of leukocytes to this critical level, the possibility that it might do so contraindicates its use in tests of this nature.

As heparin was obviously the best anticoagulant for studies of this nature, it was used in all subsequent experiments.

Comparative Effect of Blood of Different Species in Promoting Bacteriostasis.—Samples of human, rabbit, sheep, and guinea pig blood were each tested with convalescent human serum and homologous type streptococci. The number of leukocytes per c.mm. of blood were: human, 7,850; rabbit, 7,200; sheep, 5,450; and guinea pig, 6,250. Differential counts revealed a normal distribution of the

TABLE I

Comparative Effect on Bacteriostasis of Different Anticoagulants Used in Collecting Whole Blood

Convalescent serum from patient with type 6 infection	Human whole blood															
	Defibrinated				Heparin (1:16,000)				Potassium oxalate 0.12 per cent Ammonium oxalate 0.08 per cent				Sodium citrate 0.95 per cent			
	Dilution of culture of group A type 6 streptococci															
	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Undiluted serum	++	+	0	0	1	0	0	0	++±	+±	+	0	++±	++±	++	0
1:10 dilution	++	+±	9	0	9	0	0	0	++±	++	9	0	+++	++±	++	1
1:100 "	++±	++±	+	0	+	4	5	0	++±	++±	+	6	+++	++±	++	1
1:1,000 "	+++	++	++	+	+±	+	+	9	++±	++	+	+	+++	++±	++	9
Control: no serum	++++	++++	+++	+++	++++	++++	+++	++	++++	++++	+++	++	++++	+++	++±	+
Total leukocytes per c. mm.	3,150				6,200				6,900				6,750			

In all tables the degree of growth of streptococci is indicated on a ++++ to + scale; fewer than 10 colonies are represented by arabic numerals; 0 indicates no growth.

TABLE II

The Effect on Bacteriostasis of the Number of Leukocytes

Convalescent serum from patient with type 6 infection	Total leukocyte count of blood mixtures															
	6600/c. mm.				4300/c. mm.				3000/c. mm.				2000/c. mm.				1200/c. mm.			
	Dilution of culture of group A type 6 streptococci																			
	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Undiluted serum	+±	3	0	0	++	9	0	0	+++	++	+±	0	++++	++	+±	+	++++	+++	+±	+
1:10 dilution	+++	++	2	0	++++	++	++	0	++++	+++	++±	0	++++	+++	++	++	++++	+++	++	++
1:100 "	+++±	+++	+±	+	++++	++++	++	++	++++	+++	++±	+±	++++	++++	+++	++	++++	+++	+++	++
Control: no serum	++++	+++±	+++	+±	++++	++++	+++	+++±	++++	++++	+++	++	++++	+++	+++	++	++++	+++±	+++	+++

various leukocytes in each sample. Comparative measurement of complement in human, rabbit, and guinea pig plasma with hemolysin-sensitized sheep erythrocytes showed that complement was present in all samples, but in highest titer in guinea pig plasma. Sheep plasma was not tested since heterologous sensitized red blood cells were not available. With the supravital technique of Sabin (11) for observing the functional state of the polymorphonuclear leukocytes and monocytes, these cells were found to be actively motile before the test was started. Table III records the result of comparing bloods of different species, and indicates that human whole blood is far superior to that of any of the animals tested in bringing about the destruction of the streptococci by convalescent human serum. Blood from rabbits and guinea pigs younger than those used in this experiment yielded similar findings. Human blood was also effective in systems containing rabbit immune sera. Although the type 26 streptococci grew well in human, rabbit, and sheep blood to which no convalescent serum was added, their growth was partially inhibited in guinea pig blood. To eliminate the possibility that the type 26 strain used in the above experiment might be particularly resistant to phagocytosis, similar experiments were performed with types 6 and 19 streptococci and homologous convalescent sera with essentially the same result. Blood films, prepared after completion of the test from tubes containing undiluted serum, showed marked phagocytosis by human leukocytes; but only an occasional leukocyte of the blood from lower animals contained streptococci.

In order to ascertain whether systems containing both whole blood and immune serum from the same animal species, in contrast to the mixed systems reported above, were capable of promoting bacteriostasis of these streptococci, experiments were performed with normal blood and immune sera, both obtained from rabbits, and with group A streptococci of the same types used in preparing the immune sera. The results demonstrated that inhibition of growth occurred, but the rabbit whole blood was not as effective in promoting bacteriostasis as human blood even when the antibody was of rabbit origin.

Ability of Different Mixtures of Blood Cells and Plasma from Various Species to Promote Bacteriostasis.—Even though blood of other animal species was never as efficient as human blood when tested with convalescent human serum, it seemed important to find out whether mixtures of human plasma and animal blood cells or animal plasma and human blood cells would be satisfactory for bacteriostasis. Samples of whole blood from the same species as in the preceding experiment were centrifuged; the plasma was removed with fine capillary pipettes and saved for reconstitution of the different mixtures. After being thoroughly washed, the corpuscles were resuspended in plasma to the original volume and mixed carefully to avoid injuring the cells. Leukocyte counts before and after reconstitution were practically identical. Table IV again illustrates that untreated human whole blood or a mixture of

TABLE III

The Bacteriostatic Properties of Whole Blood of Different Species in the Presence of Convalescent Human Serum

Convalescent serum from patient with type 26 infection	Whole blood of various species															
	Human				Rabbit				Sheep				Guinea pig			
	Dilution of culture of group A type 26 streptococci															
	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Undiluted serum	2	0	0	0	++++	++++	++±	5	++++	++++	+++	+	++++	+++	1	0
1:10 dilution	++	++	+	0	++++	++++	++±	+±	++++	++++	+++	++	+++±	++±	2	0
1:100 "	+++	++±	+	0	++++	+++	+++	++	++++	++++	+++±	++	+++±	++±	1	0
Control: no serum	++++	+++	+++	++±	++++	++++	++++	++±	++++	++++	+++±	++±	+++	++±	+	0

TABLE IV

Bacteriostatic Properties of Mixtures of Human Plasma with Animal Blood Cells or Animal Plasma with Human Blood Cells in the Presence of Convalescent Human Serum

Convalescent serum from patient with type 26 infection	Untreated human whole blood				Human plasma and blood cells of:											
					Rabbit				Sheep				Guinea pig			
	Dilution of culture of group A type 26 streptococci															
	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Undiluted serum	0	0	0	0	++++	+++	+±	5	++++	++±	+	5	++++	+++	++	0
1:10 dilution	++	7	0	0	++++	+++	++	+	++++	+++	+++	+	++++	++++	+++	4
1:100 "	+++	++	+	0	++++	+++±	+++	+	++++	+++	+++	+	++++	++++	++±	+
Control: no serum	++++	+++	+++	++	++++	++++	+++	++±	++++	+++	+++	+	++++	+++±	+++	+

Convalescent serum from patient with type 26 infection	Human blood cells and plasma of:															
	Human				Rabbit				Sheep				Guinea pig			
	Dilution of culture of group A type 26 streptococci															
	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Undiluted serum	1	0	0	0	++++	+++	+±	0	++++	+++	++±	1	++++	++±	6	0
1:10 dilution	++	+±	0	0	+++±	+++	+++	0	+++	+++	++	0	++++	++±	+	0
1:100 "	+++	++	+	0	+++	+++	++±	+±	++++	+++	+±	+	++++	+++	+±	0
Control: no serum	++++	+++	++±	++	++++	+++	++±	+±	++++	+++	++	++	+++	+++	++	0

human blood cells and human plasma yielded the most complete bacteriostasis. Once more it can be seen that the bacteria grew well in all the control tubes with the exception of those containing the guinea pig plasma.

Supravital studies of the leukocytes of the various combinations revealed that a large proportion of the human neutrophils were viable for at least 4 hours, as indicated by their motility, contour, staining of the granules, and failure of the nuclei to stain with neutral red. Rabbit blood cells mixed with human plasma also showed little damage of the leukocytes after a 3 hour incubation period; but sheep cells showed fewer viable leukocytes; and the guinea pig leukocytes appeared least resistant to the alien human plasma.

It seems likely, but by no means certain, that the human plasma had a deleterious action on the function of the other animal polymorphonuclear leukocytes with the possible exception of those from the rabbit; but the animal plasma apparently did not adversely affect the human leukocytes. As illustrated later, some factor present in human blood, and not in that of the other species studied, is apparently required to activate a system which contains convalescent human serum.

Components of Human Blood Essential for Bacteriostatic Activity of Antibodies in Human Serum.—Because human blood was essential in testing patient's serum, an effort was made to determine which components of the blood are required for bacteriostasis of group A streptococci by antibodies in human serum. Streptococci of types 6, 19, and 26 and homologous convalescent human sera were tested with untreated human whole blood, or fresh plasma, or washed blood cells suspended either in fresh plasma or in plasma inactivated by heating at 56° C. for 30 minutes. As shown in Table V, under the conditions of the test, both leukocytes and complement are necessary for the bacteriostasis of these bacteria by the sera. It is noteworthy that no inhibition of the streptococcal growth occurred with the convalescent sera alone. The similarity in the action of untreated human whole blood and of human washed blood cells resuspended in human plasma implies that the leukocytes were uninjured by washing.

These observations are in accord with studies on streptococci and other Gram-positive bacteria by earlier investigators, and show that leukocytes, in the presence of antibody, are indispensable for the destruction of the microorganisms *in vitro*. Complement is also necessary when the test is performed within short periods of time.

Our attention was attracted to the fact that, although human leukocytes appeared normal when suspended in alien animal plasma, the streptococci grew well even though convalescent serum was present. Since complement was, of course, furnished by the plasma used, it seemed probable that factors other than complement might be required. An experiment to test this possibility is illustrated in Table VI. The convalescent human sera, types 6 and 26, were heated at 56° C. for 30 minutes to insure complete inactivation of complement. Mix-

TABLE V

Comparative Effect on Bacteriostasis of Different Fractions of Human Whole Blood

Convalescent sera from patients with:		Untreated human whole blood				Fresh human plasma				Human washed blood cells + inactivated human plasma				Human washed blood cells + fresh human plasma				Saline			
		Dilution of culture of group A type 6 streptococci																			
		10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Type 6 infection	Undiluted serum	+	4	0	0	++++	+++	+++	++±	++++	++++	++±	++±	+±	6	0	0	++++	+++±	+++±	++±
	1:10 dilution	+++	++	+	5	+++±	+++	+++	+++	++++	++++	+++±	+++	++	+±	+	4				
	1:100 "	+++	+++	++	++	++±	+++	+++	+++±	++++	+++±	+++	+++	+++	+++	++	++				
	Control: no serum	+++±	+++	++	++±	++++	+++	+++	++±	++++	+++	++±	+++	++++	+++	+++	++±	+++	+++	++±	+±
		Dilution of culture of group A type 19 streptococci																			
		10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Type 19 infection	Undiluted serum	0	0	0	0	+++±	+++±	++	+	++++	+++±	+++	++	0	0	0	0	++++	+++	++±	+±
	1:10 dilution	1	0	0	0	+++±	+++	++	+±	+++±	+++	+++	++±	4	0	0	0				
	1:100 "	++±	0	0	0	+++±	+++	+++	++±	++++	+++	+++	++	+++	9	0	0				
	Control: no serum	+++±	+++±	+++	++±	++++	+++	+++	+++	++++	+++	+++	+±	++++	+++	+++	+++	++++	+++	+++	+±
		Dilution of culture of group A type 26 streptococci																			
		10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Type 26 infection	Undiluted serum	2	0	0	0	+++	++±	++±	0	++++	+++	++	++	2	1	0	0	++++	+++	+++	++
	1:10 dilution	++	5	0	0	+++	+++	++	+	++++	++++	++++	+±	+±	7	0	0				
	1:100 "	+++	++±	7	0	+++	++±	++	+	++++	++++	++++	+±	+++	++	+	0				
	Control: no serum	++++	+++	+++	++±	++++	+++	+++	++	++++	+++	+++	++	++++	+++	+++	++	++++	+++	+++	++

TABLE VI

Demonstration of a Thermostable Factor in Human Plasma Necessary for Bacteriostasis

Inactivated convalescent sera from patients with:	Human washed blood cells															
	+ inactivated human plasma + fresh guinea pig plasma				+ inactivated human plasma				+ fresh guinea pig plasma				+ fresh human plasma			
	Dilution of culture of group A type 6 streptococci															
	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Type 6 infection: Undiluted serum	++	+	0	0	+++±	+++	+±	8	+++±	+++	++	2	+	8	0	0
Type 6 infection: Control: no serum	++++	+++	++±	9	++++	+++	++±	++	++++	+++	++	4	++++	+++	++±	++
	Dilution of culture of group A type 26 streptococci															
	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Type 26 infection: Undiluted serum	1	1	0	0	++++	+++	++	+	++++	+++	+++	9	+±	0	0	0
Type 26 infection: Control: no serum	++++	+++	++	+±	++++	+++	+++	++±	++++	+++	++±	+	++++	+++	++	++

TABLE VII

Comparative Complement Activity of Different Species in the Bacteriostatic Test

Inactivated convalescent sera from patients with:	Human washed blood cells + inactivated human plasma															
	+ fresh human plasma				+ fresh guinea pig plasma				+ fresh rabbit plasma				+ fresh sheep plasma			
	Dilution of culture of group A type 19 streptococci															
	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Type 19 infection: Undiluted serum	+	0	0	0	++	4	0	0	++++	+++	+	3	++++	++++	++±	0
Type 19 infection: Control: no serum	++++	+++	+++	++±	++++	++++	+++	4	++++	++++	++±	++±	++++	++++	+++	4
	Dilution of culture of group A type 26 streptococci															
	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Type 26 infection: Undiluted serum	+±	0	0	0	++±	+	2	0	++++	+++	+±	6	++++	++++	+++	6
Type 26 infection: Control: no serum	++++	++++	+++	++	++++	+++	++±	+±	++++	+++	+++	+±	++++	+++	+++±	+

tures of blood cells and plasma were prepared as in previous experiments. The combination of 3 parts of inactivated human plasma with 1 part of fresh guinea pig plasma equalled in volume the amount of plasma used in the other samples. In this system and in that containing fresh human plasma, bacteriostasis was obtained in the presence of human washed blood cells. In those containing inactivated human plasma without added complement or fresh guinea pig plasma, *i.e.*, complement without human plasma, little or no activity was observed. From this observation one may deduce that, in addition to complement and leukocytes, a thermostable factor in human plasma is apparently required for the phagocytosis of the streptococci sensitized by convalescent human serum.

Further investigation demonstrated that this thermostable factor was present in both human serum and plasma. It was effective in 1:12 dilution of fresh human plasma and was destroyed by heating at 70° C. for 30 minutes; moreover, it persisted for 7 weeks, though diminished, in serum kept at 4° C. The demonstration of this relatively thermostable component in human plasma suggested that one might compare the activation of bacteriostatic antibodies by the complement of different animal species. Complement in rabbit or sheep plasma was found to be less potent than that of guinea pig or man in this respect (Table VII). Although complement may behave differently in bacteriostatic and hemolytic systems, these observations in general agree with those of Capart (12), who evaluated quantitatively the activity of complement for lysing sensitized heterologous and homologous red blood cells, and found that guinea pig was the greatest, sheep the least, and human and rabbit intermediate. The complement activity of fresh human plasma does not necessarily account for the increased bacteriostasis in this experiment (Table VII) over that in the system containing rabbit plasma, since the addition of human plasma has also augmented the thermostable component and may possibly be responsible for the more effective streptococcal stasis.

These data indicate that, among the various species studied, only man supplies in his blood all components essential for destroying group A streptococci in the presence of homologous convalescent human serum.

DISCUSSION

From the data herein presented, it is shown that in the method of indirect bacteriostasis described, the most satisfactory anticoagulant for blood is heparin. The complement-fixing action of heparin, reported by Wising (6), is insufficient to interfere with this test; and the method is best standardized by its use. The danger of lowering the number of leukocytes below the level for effective phagocytosis contraindicates defibrination as a method for preventing the coagulation of blood in these bacteriostatic tests. Although calcium-neutralizing agents are unsatisfactory, their deleterious effect on phago-

cytosis is not fully understood. The most plausible explanation appears to be a reduction in the ionizable calcium. An additional factor, the formation of a precipitate, must be considered in oxalated blood. The calcium oxalate particles may be taken up by the leukocytes which would subsequently be unable to engulf the streptococci. The mechanism by which such precipitates interfere with phagocytosis is the subject of the subsequent communication (13).

It was observed that blood of rabbit, guinea pig, or sheep could not be substituted for human blood when convalescent human sera supplied the antibody; nor could animal leukocytes or plasma from these other animal species replace the corresponding portion of human blood. The reasons for this specificity are not, however, entirely clear. A deleterious effect of human plasma on animal leukocytes has been suggested by supravital studies, and this probably explains their inactivity when suspended in human plasma. When studied by the supravital technique, the guinea pig leukocytes were either severely damaged or obviously dead very shortly following their suspension in human plasma; the sheep leukocytes were less affected; and most of the rabbit neutrophils appeared moderately active for at least 3 hours, although their phagocytic function may have been somewhat diminished. It is also possible that in those tests utilizing animal blood the convalescent human serum, although making up only one-seventh of the contents of each tube, may nevertheless have had a toxic action upon the animal leukocytes. Human leukocytes on the contrary did not appear to be adversely altered by alien plasma.

The human cell-animal plasma mixtures probably failed to promote bacteriostasis because of the absence of a relatively thermostable factor present in human blood. In addition to this thermostable factor, complement and leukocytes were necessary for the destruction of the streptococci. Ward and Enders (2) have shown that complement accelerates the sensitization of pneumococcus cells by homologous antibody, which in turn makes it possible for them to be phagocytized by leukocytes; a similar process probably occurs in the bacteriostasis of streptococci described in the present study. How the relatively thermostable factor functions is not known. It was, however, apparent that, if complement in guinea pig plasma was added to the thermostable component, good bacteriostasis resulted; but if rabbit or sheep plasma, which contains less complement than guinea pig, was substituted, the bacteriostasis was much less.

When guinea pig plasma and homologous or heterologous blood cells were employed, some inhibition of streptococcal growth occurred in the absence of convalescent serum. This phenomenon was noted repeatedly and may conceivably be due to some greater resistance of guinea pigs for group A streptococci as compared with that of the other species investigated.

From these observations it is evident that in order to demonstrate

bacteriostasis of group A streptococci when the antibody is of human origin, a relatively thermostable factor present in human plasma or serum is required in addition to human leukocytes and to complement, preferably of human origin. Of the blood from the various species studied, therefore, only human whole blood supplies all the elements necessary for bacteriostasis in the presence of convalescent human serum.

SUMMARY

1. Heparin was more satisfactory for preventing blood from clotting than defibrination, potassium and ammonium oxalate, or sodium citrate in bacteriostasis of group A streptococci in the presence of streptococcal antibodies in convalescent serum.

2. Blood from rabbit, guinea pig, or sheep could not be substituted for human blood in promoting bacteriostasis when human antibody was used. Mixtures of human leukocytes and plasma of each of these animals or of animal leukocytes and human plasma were also not effective with human antibody.

3. Complement, leukocytes, and a thermostable factor which was found in human plasma were essential in the indirect bacteriostatic technique employed for the inhibition of streptococcal growth in the presence of convalescent human serum.

4. The thermostable component was active in human serum, as well as in plasma, in 1:12 dilution, withstood storage at 4° C. for at least 7 weeks, and was destroyed by heating at 70° C. for 30 minutes.

BIBLIOGRAPHY

1. Rothbard, S., *J. Exp. Med.*, 1945, **82,** 93.
2. Ward, H. K., and Enders, J. F., *J. Exp. Med.*, 1933, **57,** 527.
3. Dingle, J. H., Fothergill, L. D., and Chandler, C. A., *J. Immunol.*, 1938, **34,** 357.
4. Hamburger, H. J., Physikalesch-chemische Untersuchungen über Phagocyten, Weisbaden, J. F. Bergman, 1912.
5. Colebrook, L., and Storer, E. J., *Brit. J. Exp. Path.*, 1924, **5,** 47.
6. Wising, P. J., *Acta. med. Scand.*, 1937, **91,** 550.
7. Ward, H. K., *J. Exp. Med.*, 1930, **51,** 675.
8. Fleming, A., *Brit. J. Exp. Path.*, 1926, **7,** 281.
9. Thalhimer, W., and Colwell, C., *J. Lab. and Clin. Med.*, 1929, **14,** 441.
10. Kuttner, A. G., and Lenert, T. F., *J. Clin. Inv.*, 1944, **23,** 151.
11. Sabin, F. R., *Bull. Johns Hopkins Hosp.*, 1923, **34,** 277.
12. Capart, J., *Compt. rend. Soc. biol.*, 1928, **98,** 1250.
13. Rothbard, S., *J. Exp. Med.*, 1945, **82,** 119.