PNEUMOCOCCUS CULTURES IN WHOLE FRESH BLOOD.

I. The Retardative Effect of the Blood of Immune Animals and the Mechanism of the Phenomenon.

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The failure to realize more fully in the field of specific immunotherapy the expectations that were engendered by the discovery of specific antitoxins for diphtheria and tetanus is due, no doubt, in large measure to the lack of definite and complete knowledge of the mechanisms of immunity reactions.

It is noteworthy that, with few exceptions, a multiplicity of theories has been evolved with respect to the identity and nature of the factors and processes operative in natural recovery from each infectious disease to which man is subject. This is true of infections by the pneumococcus, notwithstanding the fact that these infections lend themselves, to an exceptional degree, to experimental study. It would be difficult to mention a more striking example of antibacterial immunity than that conferred upon a susceptible animal by the administration of antipneumococcus serum. Moreover, some of the species of laboratory animals are highly susceptible to pneumococcus infections, while others are refractory to a corresponding degree, thus making it possible to study any type of immunity—natural immunity or active or passive acquired immunity.

The protective power of antipneumococcus serum has been ascribed to different factors by various investigators. Thus Mennes,[1] Boehncke and Mouriz-Riesgo,[2] and others advanced the theory that antipneumococcus serum possesses marked

[1] Mennes, F., *Z. Hyg. u. Infectionskrankh.*, 1897, xxv, 413.

[2] Boehncke, K. E., and Mouriz-Riesgo, J., *Z. Hyg. u. Infectionskrankh.*, 1915, lxxix, 355.

antitoxic properties, while Neufeld and Rimpau[3,4] claim that pneumococcus immunity depends entirely upon the bacteriotropic substances of the immune serum and the phagocytic cells of the host. More recently it has been pointed out by Wright[5] that the whole uncoagulated blood of man is, in certain instances, highly pneumococcidal, and a case is cited in which 1 cc. of blood killed from 600,000 to 1,000,000 organisms. Heist and his collaborators[6,7] have extended Wright's observations, including experimental animals. It is claimed by them that the pneumococcidal capacity of the whole uncoagulated blood is a direct index of resistance to pneumococcus infection and that the virulence of the organisms is directly proportional to their insusceptibility to this killing property of the blood. Cole and his coworkers[8] and Winternitz and Kline[9] believe that any or all of the factors mentioned above cannot be held entirely responsible for immunity to pneumococcus infection and that an unidentified factor is essential.

The work reported in the present paper concerns the alleged pneumococcidal property of the whole blood of immune animals and it is therefore desirable to state briefly a few points concerning antipneumococcus serum which are of interest in this connection. Pneumococci can be grown for an unlimited number of generations in the most potent antipneumococcus serum. This treatment profoundly affects the organisms, as shown by Stryker,[10] yet there is no evidence that any of the organisms are killed by the serum. The same is true of the blood serum of naturally immune animals. Pigeons, for example, possess practically an absolute immunity to pneumococcus infections, while the fresh serum of pigeons is a good culture medium for pneumococci. The defibrinated blood of immune animals is only a slightly less favorable medium than the serum. On the other hand, pneumococci do not multiply in the blood stream of refractory animals and the intravenous administration of antipneumococcus serum sterilizes,

[3] Neufeld, F., and Rimpau, W., *Deutsch. med. Woch.*, 1904, xxx, 1458.

[4] Neufeld, F., and Rimpau, W., *Z. Hyg. u. Infectionskrankh.*, 1905, li, 283.

[5] Wright, A. E., On pharmo-therapy and preventive inoculation applied to pneumonia in the African native, New York, 1915, 78.

[6] Heist, G. D., Solis-Cohen, S., and Solis-Cohen, M., *J. Immunol.*, 1918, iii, 261.

[7] Heist, G. D., and Solis-Cohen, S., *J. Immunol.*, 1919, iv, 147.

[8] Avery, O. T., Chickering, H. T., Cole, R., and Dochez, A. R., Acute lobar pneumonia. Prevention and serum treatment, Monograph of The Rockefeller Institute for Medical Research, No. 7, New York, 1917.

[9] Winternitz, M. C., and Kline, B. S., *J. Exp. Med.*, 1915, xxi, 320.

[10] Stryker, L. M., *J. Exp. Med.*, 1916, xxiv, 49.

within a few minutes, the blood of an animal having a pneumococcemia. This last phenomenon has been ascribed to the agglutinating and bacteriotropic properties of the immune serum and the phagocytic cells of the host rather than to a bactericidal action of the serum.[11] Since the reactions here concerned take place within the whole blood, it would, in the light of the reports of Wright and Heist and his coworkers, be reasonable to suppose that the bactericidal property of the whole circulating blood of the immune animal plays an important part in this phenomenon.

EXPERIMENTAL.

Technique of the Whole Blood Test.[5, 6]—Sets of five or more capillary glass tubes about 1 mm. in diameter are filled respectively, by capillary attraction, to a fixed point with graded dilutions of an 18 hour blood broth culture of pneumococci, the culture being immediately drawn from the tubes by touching the filled ends with sterile gauze. This leaves a coating of culture on the walls of the tubes, the number of bacteria left behind varying with the dilution of the culture. When the tubes have dried in the air for a few minutes they are filled to the fixed point with the blood to be tested by touching them to a drop of blood as it flows from a punctured vein. The tubes are sealed by dipping them into melted paraffin (both ends may be sealed, the loaded end first). They are incubated at 37°C. for any desired length of time and then examined by making films of the contents, staining, and examining with the microscope, or by making cultures in suitable medium. It is expedient to make five or more dilutions of the culture, varying from undiluted culture to a dilution of 1 : 1,000, broth being used as the diluting fluid. Three or more sets of capillary tubes should be prepared for each test in order that a set of dilutions may be examined after different incubation periods, varying from 1 to 96 hours as the test may demand.

For microscopic examination the entire contents of a tube are blown upon a slide and spread thinly over the surface. The films are allowed to dry in the air, fixed in methyl alcohol, and stained with Manson's stain. To avoid overlooking the bacteria, especially when they are not evenly distributed, it is necessary to go over the whole preparation with great care. When diplococci are found in the preparations it indicates that multiplication has occurred, for on control slides made immediately after the tubes are loaded, bacteria are never found, except when undiluted culture has been used to charge the

[11] Bull, C. G., *J. Exp. Med.*, 1915, xxii, 457.

tubes, and even here the slides are usually negative. Cultures are made from the tubes by blowing the contents into the condensation fluid of freshly prepared rabbit blood agar slants, and the clot, when whole blood is being tested, is broken up and spread over the surface of the slant. The inoculated tubes are examined after from 24 to 48 hours incubation.

In determining the relative effects of the blood of different animals on the growth of the culture it is essential that the tests should be prepared simultaneously and with the same dilutions of culture. All other conditions of the tests must be the same in each instance, for slight variations will give incorrect results. It is well to run a control set of tubes with normal rabbit blood in order to establish the viability and vigor of the culture.

Any digression from these general directions for technique will be pointed out in the protocols.

The distinctive and important feature of the tests is that the organisms are brought into contact with the undiluted blood or other fluid to be tested.

Experiment 1.—In these tests with pigeon blood the capillaries were charged with dilutions of culture ranging from undiluted culture to a dilution of 1 : 625. Sets of tubes were examined after from 1 to 72 hours incubation. Control tests with normal rabbit blood were made in each instance. Fresh pigeon serum was also used as a control.

Pigeon blood, as compared with rabbit blood, greatly retards the multiplication of pneumococci. Capillaries charged with undiluted culture and filled with rabbit blood are positive on microscopic examination after from ½ to 1 hour incubation, while those filled with pigeon blood do not become positive until after an incubation period of from 6 to 18 hours. Capillaries with a 1 : 625 dilution of culture become positive in rabbit blood after from 5 to 7 hours incubation, while in the tubes filled with pigeon blood organisms cannot be found until after about 72 hours. Tubes with intermediate dilutions of culture become positive after a corresponding incubation period. All the tubes give positive cultures at any time during the test (Table I).

A negative microscopic examination does not necessarily mean that the pneumococci have been killed, but a negative culture is essential to such a conclusion. As far as the rabbit and pigeon are concerned, the rapidity with which the cultures develop may be taken as a fair index of their relative susceptibility to infection by these organisms.

TABLE I.

Pneumococcus Cultures in Fresh Pigeon Blood; Microscopic versus Culture Test.

Dilution of culture with which capillary was charged.	Incubation.	Microscopic examination.	Culture on rabbit blood agar.
	hrs.		
Undiluted.	1	−	+
1 : 5	1	−	+
1 : 25	1	−	+
1 : 125	1	−	+
1 : 625	1	−	+
Undiluted.	6	−	+
1 : 5	6	−	+
1 : 25	6	−	+
1 : 125	6	−	+
1 : 625	6	−	+
Undiluted.	12	+	+
1 : 5	12	−	+
1 : 25	12	−	+
1 : 125	12	−	+
1 : 625	12	−	+
Undiluted.	18	+	+
1 : 5	18	+	+
1 : 25	18	−	+
1 : 125	18	−	+
1 : 625	18	−	+
Undiluted.	42	+	+
1 : 5	42	+	+
1 : 25	42	+	+
1 : 125	42	+	+
1 : 625	42	−	+
Undiluted.	65	+	+
1 : 5	65	+	+
1 : 25	65	+	+
1 : 125	65	+	+
1 : 625	65	+	+

Experiment 2.—Comparative tests were made with the blood of a number of other laboratory animals. The microscopic method was used to determine whether growth had occurred, a positive result meaning that diplococci were found in the stained preparations. Sufficient cultures were made to eliminate the possibility of contaminations. Since the tubes always became positive in the order of the dilutions of culture with which they had been charged, only the results of the two extreme dilutions are given. Table II gives a summary of these tests.

Table II includes only normal individuals of the different species which are arranged in the order of the incubation (latent) period of

TABLE II.

Latent Period of Pneumococcus Cultures in the Whole Blood of Different Animals.

Animal.	Length of time after which cultures are positive on microscopic examination.	
	Capillaries charged with undiluted culture.	Capillaries charged with 1:625 dilution of culture.
	hrs.	*hrs.*
Rabbit	½–1	3–5
Mouse	½–1	4–6
Cat	1–2	5–6
Guinea pig	1–2	6–7
Sheep	2–3	6–7
Man	2–3	6–7
Dog	3–4	8–12
Hen	4–5	12–15
Pigeon	6–12	48–72

pneumococci in the respective bloods. It is seen that the rabbit, mouse, and cat come at the top of the list, the pigeon, hen, and dog at the other end, while the guinea pig, sheep, and man occupy intermediate positions. There is no doubt that in a general way this is the order of susceptibility to infection of these species to the pneumococcus, the rabbit and mouse being the most susceptible and the pigeon and hen the most resistant; but the relative positions of those falling in the middle zone are questionable. The cat, for example, should be farther separated from the rabbit and mouse, particularly on intravenous inoculation. Further tests showed that the method is not sensitive enough to classify normal individuals of the same species

with respect to susceptibility to pneumococcus infection. Experiments on vaccinated rabbits showed, however, that in this instance, an immunity reaction which is not made evident by other methods used to demonstrate antibodies[5, 7] can be detected by this method. The data recorded in Experiment 3 illustrate this point.

Experiment 3.—Each of two rabbits was given intravenously the killed cultures from two blood agar slants of pneumococci. On the 3rd day after the injection the rabbits were bled and the sera tested in the ordinary way for agglutinins and opsonins. Whole blood tests were set up at the time the blood was collected for the serum. The tests were repeated on each succeeding day, corresponding tests being made on normal rabbits as controls. Even on the 3rd day the blood of one of the treated rabbits caused a slight delay in the growth of the culture. On the 4th day the blood of both rabbits caused a definite lengthening of the latent period and this effect rapidly increased on the succeeding days, while agglutinins and opsonins were not demonstrable in the serum, by ordinary methods, until the 6th day, although 50 per cent serum was present in the tests.

As may be inferred from Experiment 3, active immunization of rabbits against pneumococci confers upon their blood the property of retarding the growth of these organisms, the actively immune rabbit conforming to the normal pigeon in this respect. Further experiments showed that passive immunity gives identical results. Guinea pigs and rabbits were used in these experiments.

The foregoing experiments show that, in a general way, the rapidity with which pneumococci multiply in the fresh whole blood of various animals is directly proportional to the susceptibility of these animals to infection by the pneumococcus. Slight differences in susceptibility cannot, at least in every instance, be detected by this method. The cat, for example, is many times more resistant than the rabbit to infection with pneumococci on intravenous inoculation, while the whole blood test would indicate that the cat is only slightly more resistant. It is also shown that natural and artificial immunity —both active and passive—are similar with respect to this test. Finally, the conclusion that the fresh uncoagulated blood of immune animals is highly pneumococcidal[5, 6, 7] is doubtful, since the mixtures did not become sterile during the course of the experiments and multiplication of the organisms always occurred when incubation was sufficiently prolonged. On the other hand, it cannot be concluded

that no killing occurs; but to prove positively that the blood possesses a pneumococcidal property it would be essential that some of the pipettes, for example those charged with the higher dilutions of culture, should become free of viable organisms. The phenomenon described is, however, closely associated with, and at least roughly parallel to, resistance to infection. It was thought, therefore, that the working out of the mechanism of the reaction would possibly give important information as to the nature of the factors and processes involved in pneumococcus immunity. A large number of experiments have been carried out with this end in view.

Microscopic Study.

A microscopic study was made of cultures of pneumococci at different stages of development in the whole blood of the animals used in the foregoing experiments. Capillary tubes were charged with the culture and loaded with the fresh uncoagulated blood according to the method already described. Sufficient tests were prepared in each instance to permit of frequent examinations. Cultures made in the blood of normal rabbits were used as controls, rabbit blood being an excellent medium for pneumococci. The following points were noted: (1) the kind of clot formed by the different bloods, (2) distribution of diplococci in the medium, (3) chain and clump formation, and (4) phagocytosis.

Clot Formation.—The bloods of different species behave differently with respect to retraction of the clot within the capillary tubes. The rabbit clot retracts both transversely and longitudinally, usually pulling away from the walls of the tube within a short time. This results in a small clot, and a relatively large amount of free serum. The pigeon clot retracts very little in any direction and usually continues to fill the whole tube, and there is only a small amount of free serum. The hen clot usually retracts longitudinally, but less than that of rabbit blood, and pulls away from the walls of the tube to a much less degree than the latter. The other bloods come between these extremes with respect to retraction of the clot. This point will be referred to later, since it apparently plays at least a secondary part in the phenomenon under investigation.

Distribution of the Organisms in the Culture.—Three films were made from each capillary culture, (1) from the free serum, (2) from the serum adhering to the clot, and (3) from the clot. The serum was removed from the small culture tubes by means of a very fine capillary. The clot was blown upon a slide and moved over the surface in order to make a film of the serum adhering to the clot. The clot was then dried on filter paper and a preparation made of the dried clot. These preparations were made at all stages of the development of the cultures and it was found that in the earlier stages the pneumococci were multiplying actively in the serum, fewer organisms were in the serum adhering to the clot, and the clot itself was not invaded until the cultures were well developed. This was particularly true of the capillaries containing immune blood—pigeon, hen, or immune rabbit blood. The clot of normal rabbit blood was invaded earlier in the development of the culture. This distribution might have been predicted because (1) the seeding organisms are at the periphery of the clot, and (2), as has been stated, pneumococci grow readily in the most potent immune serum.

Chain and Clump Formation.—Just as in immune serum, pneumococci grow in chains and clumps in immune blood, the growth in this instance being largely in the serum after coagulation has taken place. In pigeon blood there is only a slight clumping but long chains are formed, while in hen blood large clumps develop and also long chains. Chains and clumps are formed in immune rabbit blood, the size of the clumps and the length of the chains varying with the degree of immunity and the incubation time. The first indication of the development in rabbits of the immunity response to an inoculation of the organisms is chain formation, and the second, clump formation. In highly immune blood, the growth is restricted to chains and clumps until the culture approaches maturity when the growth may become diffuse (uniformly distributed diplococci) within a short time. The chain and clump phases of the cultures remind one of local infections and the diffuse phase of general infections. Apparently growth does not become diffuse until the antibodies of the blood have been exhausted, or until the organisms have become less susceptible to the antagonistic influence of the blood.[12] The chain and clump phenom-

[12] Bull, C. G., *J. Exp. Med.*, 1916, xxiv, 7.

ena occur in pigeon blood, hen blood, in the blood of an immune rabbit or any other immunized animal, and to a less degree in normal dog blood. They do not occur in normal rabbit, mouse, or cat blood. This fact must be kept in mind when making microscopic examination of cultures in immune blood; otherwise, even massive clumps may be overlooked and the specimen pronounced negative.

Phagocytosis.—Phagocytosis of the pneumococci was first observed in preparations made from cultures in hen blood. For this purpose the cultures should be examined after from 4 to 5 hours incubation. Preparations from the serum adhering to the clot contain the greatest number of phagocyting cells (polymorphonuclear leucocytes). Phagocytosis occurs in any immune blood. In order to demonstrate this appearance to advantage in highly immune bloods (pigeon, immune rabbit) it was necessary to charge the tubes with concentrated suspensions of the organisms. This was effected by centrifugalization and resuspension of broth cultures. A tenfold concentration gave good results. When more dilute cultures are used, the number of phagocyting cells is small and satisfactory observations cannot be made.

The tentative hypothesis which we based on the above observations was that the development of the culture to the point of being positive on microscopic examination was retarded by chain and clump formation and possibly by phagocytosis. When the organisms are held tightly in a few clumps, instead of being uniformly distributed through the medium, there is less probability of observation with the microscope. Thus, of two preparations containing the same number of organisms, one clumps and the other evenly distributed diplococci, the first, barring accident, could easily be found negative and the second positive. Moreover, multiplication is probably actually retarded because of reduced nutrition or an accumulation of inhibitory substances in the interior of the clump. In regard to phagocytosis the notion was that either a certain number of the pneumococci were killed by the phagocytes or multiplication within the phagocytes was held in check for a time, thus retarding the development of the culture. Further work was planned to determine the part played by the leucocytes in the reaction.

Phagocytosis and Retardation of Growth.

If phagocytosis is essential in the retardation of the cultures in immune blood, it is evident that the phenomenon depends on two agents; *viz.*, opsonins and leucocytes. The reaction should not occur in the absence of either factor, and one of the factors being constant the retardation should be directly proportional, within certain limits, to the quantity of the other factor present. This conception of the problem guided the experiments recorded below.

Defibrinated Blood.—It is stated by Heist and his associates[6] that defibrinated immune blood (chicken) is as good a medium for pneumococci as defibrinated normal rabbit blood. We studied this point since it appeared that phagocytosis does not play an essential part in the retardation of the growth of the culture, since the leucocytes are present in defibrinated blood, presumably, as in whole blood. Immune rabbit blood was used in this study.

Experiment 4.—Capillary tubes were prepared as usual with (1) whole blood, (2) defibrinated blood, and (3) serum. The tests were set up simultaneously, the capillaries having been charged with the same dilutions of culture. The defibrinated blood and serum tests were prepared a few minutes after the blood had been collected by cardiac puncture. The defibrination was effected by shaking the blood with glass beads and the serum was collected by centrifugalizing a portion of the blood immediately after coagulation. The whole blood was collected from the ear vein.

By examining the cultures at short intervals it was found that the pneumococci multiply more rapidly in the defibrinated blood than in the whole blood, but growth in the serum is still more rapid than in the defibrinated blood (Table III).

Leucocytes of Defibrinated Blood.—Whole and defibrinated blood from the same animal were studied in regard to the number, type, and condition of the leucocytes present, total and differential counts being made. It was found that defibrination removes from one-half to three-fourths of the total leucocytes, and a large percentage of those remaining in the defibrinated blood are lymphocytes. In one instance the following observations were made: whole blood, total count 12,515, polynuclear cells 7,460, lymphocytes 4,880, mononuclear leucocytes 175; defibrinated blood, total count 3,900, polynuclear cells 835, lymphocytes 2,905, mononuclear leucocytes 160. In other instances the total count was not reduced to such a low figure but the

number of polynuclear cells remaining in the defibrinated blood was always relatively very low. It was further noted that the process of defibrination visibly injured a large percentage of the polynuclear cells.

In these experiments there was, at least roughly, a mathematical relation between the number of polynuclear cells present and the rate

TABLE III.

Pneumococcus Cultures in the Whole Blood, Defibrinated Blood, and Serum of an Immunized Rabbit and in Normal Blood.

Dilution of culture with which capillary was charged.	Incubation.	Microscopic examination.			
		Whole blood.	Defibrinated blood.	Serum.	Normal rabbit blood.
	hrs.				
Undiluted.	4	−	+	+	+
1 : 5	4	−	−	+	+
1 : 25	4	−	−	−	+
1 : 125	4	−	−	−	+
1 : 625	4	−	−	−	−
Undiluted.	8	+	+	+	+
1 : 5	8	−	+	+	+
1 : 25	8	−	+	+	+
1 : 125	8	−	−	+	+
1 : 625	8	−	−	−	+
Undiluted.	12	+	+	+	
1 : 5	12	+	+	+	
1 : 25	12	+	+	+	
1 : 125	12	−	+	+	
1 : 625	12	−	−	+	

of multiplication of the pneumococci. The points determined also eliminate the red cells as a decisive factor in the reaction.

Relation between the Number of Cells Present and Retardation of Growth.—The experiments given here corroborate the conclusion drawn from the study of the defibrinated blood; *i.e.*, the latent period of the culture is directly proportional to the number of leucocytes present.

Experiment 5.—Blood was collected from immune animals (immunized rabbits and hens) in oiled glassware, quickly centrifugalized at high speed, and the three layers (plasma, leucocytic cream, and cells) were separated, remixed in different proportions, and the mixtures run into culture-charged capillary tubes before coagulation occurred. Washed guinea pig leucocytes and plasma or serum mixtures were also tested.

TABLE IV.

Pneumococcus Cultures in Hen Blood, Serum, Plasma, and Equal Parts of Plasma and Leucocytic Cream.

Dilution of culture with which capillary was charged.	Incubation.	Microscopic examination.			
		Whole blood.	Serum.	Plasma.	Equal parts of plasma and leucocytic cream.
	hrs.				
Undiluted.	4	+	+	+	+
1 : 5	4	+	+	+	−
1 : 25	4	−	+	+	−
1 : 125	4	−	−	−	−
1 : 625	4	−	−	−	−
Undiluted.	8	+	+	+	+
1 : 5	8	+	+	+	+
1 : 25	8	+	+	+	−
1 : 125	8	+	+	+	−
1 : 625	8	−	+	+	−
Undiluted.	12	+			+
1 : 5	12	+			+
1 : 25	12	+			+
1 : 125	12	+			−
1 : 625	12	+			−
Undiluted.	24				+
1 : 5	24				+
1 : 25	24				+
1 : 125	24				+
1 : 625	24				+

The following observations were made (Tables IV and V). (1) Pneumococci multiply rapidly in both the serum and coagulated plasma of immune animals, in some instances growth being more rapid in the plasma than in the serum. (2) Plasma and cells mixed in equal amounts inhibit growth to the same degree as the original whole blood. (3) A mixture of equal quantities of plasma and leucocytic cream delays the development of the culture more than the original whole blood.

TABLE V.

Pneumococcus Cultures in Hen Blood, Plasma, Sedimented Cells, and Ground Sedimented Cells.

Dilution of culture with which capillary was charged.	Incubation.	Microscopic examination.			
		Whole blood.*	Plasma.	Sedimented cells.†	Ground sedimented cells.
	hrs.				
1 : 10	11	+	+	−	+
1 : 100	11	+	+	−	+
1 : 1,000	11	−	+	−	+
1 : 5,000	11	−	+	−	+
1 : 10,000	11	−	−	−	−
1 : 10	28	+	+	−	+
1 : 100	28	+	+	−	+
1 : 1,000	28	+	+	−	+
1 : 5,000	28	+	+	−	+
1 : 10,000	28	−	+	−	+
1 : 10	58	+		+	
1 : 100	58	+		−	
1 : 1,000	58	+		−	
1 : 5,000	58	+		−	
1 : 10,000	58	+		−	
1 : 10	72			+	
1 : 100	72			+	
1 : 1,000	72			+	
1 : 5,000	72			−	
1 : 10,000	72			−	
1 : 10	96			+	
1 : 100	96			+	
1 : 1,000	96			+	
1 : 5,000	96			+	
1 : 10,000	96			+	

* The hen was given 10 cc. of antipneumococcus serum 30 minutes before the blood was collected.

† Cultures of the sedimented cells on rabbit blood agar were positive throughout the experiment.

(4) Plasma plus washed leucocytes delays growth. (5) Immune serum and leucocytes do not retard growth to the same degree as plasma and leucocytes. (6) The compact cells[13] of a highly immune blood produce a prolonged latent period in the cultures which do not become positive on microscopic examination until after from 48 to 72 hours. (7) If the cells, as described under (6), are thoroughly ground with a mortar and pestle in fine, sterile sand, the pneumococci multiply as rapidly as in the serum or plasma. This is also the case when leucocytic cream or leucocytes are used in making the mixtures.

Here again it is indicated that the presence of leucocytes is essential to the retarding property of the blood, and since a mechanical destruction of the phagocytes, nothing being removed or added, robs the plasma-cell mixtures of their retarding property, it seems to be established that the act of phagocytosis plays a decisive part in the reaction. It remains to be explained, however, why a mixture of leucocytes and immune serum does not retard the development of the culture to the same extent as the plasma of the animal plus an equal number of leucocytes. A mechanical factor is involved here. In the serum-leucocyte mixtures the leucocytes quickly settle to the bottom of the capillary, leaving a layer of serum in which the pneumococci rapidly multiply. It should be stated here that when chicken plasma is prepared as described above the clot does not retract in the tubes but continues to occupy the whole tube, thus bringing the leucocytes into direct contact with the pneumococci which have been deposited on the walls.

Concentration of Antibodies and Retardation of Growth.

Experiment 6.—A series of guinea pigs was given intravenous injections of varying quantities of antipneumococcus horse serum, the amount of serum injected ranging from 1 to 5 cc. 2 hours later capillary cultures were made of the whole blood of the guinea pigs. A normal guinea pig was included in the series. A set of the cultures was examined every 2 hours.

The latent period of the cultures was directly proportional to the quantity of serum injected, the cultures in the blood of the guinea pig which received 1 cc. of serum having a longer incubation period than those in normal blood, while the cultures made in the blood of the animal which received 5 cc. of serum were the last to develop (Table VI).

[13] The cells were sedimented by centrifugalization, but sufficient serum to opsonize the bacteria still adhered to the cells.

TABLE VI.

*Pneumococcus Cultures in the Blood of Passively Immunized Guinea Pigs.**

Dilution of culture with which capillary was charged.	Incubation.	Microscopic examination.		
		Guinea Pig A (1 cc. of immune serum).	Guinea Pig B (5 cc. of immune serum).	Guinea Pig C (normal control).
	hrs.			
Undiluted.	4	+	−	+
1 : 5	4	−	−	+
1 : 25	4	−	−	+
1 : 125	4	−	−	−
1 : 625	4	−	−	−
Undiluted.	8	+	+	+
1 : 5	8	+	−	+
1 : 25	8	+	−	+
1 : 125	8	+	−	+
1 : 625	8	−	−	+

* The antipneumococcus serum was given intravenously 2 hours before the cultures were made.

DISCUSSION.

The observations outlined above indicate that the whole blood of immune animals merely retards the multiplication of pneumococci instead of killing them, as is claimed by Wright and Heist and his coworkers. It is clearly demonstrated that failure to develop within 24 hours to the point of being positive on microscopic examination does not prove that the organisms with which the cultures were seeded have been killed. We have observed the development of cultures after a latent period of 72 hours. The authors referred to above ended their experiments after an incubation period of 24 hours. Should the organisms never multiply in the original medium, it could not be concluded that they had been killed, unless the cultures were proved to be sterile by failure of growth when transferred to a highly favorable medium. Even in this instance, particularly after a prolonged original incubation period, the possibility of involution would not be eliminated. It is claimed by Heist and his collaborators that a negative microscopic examination after 24 hours incubation invariably means pneumococcidal effect. The sterility test employed, however, was that of blowing the contents of the capillary tubes into broth.

The small number of organisms thus transferred was probably unable to overcome the resistance of the relatively large volume of liquid medium. In our experiments fresh rabbit blood agar tubes were substituted for the broth.

In every instance so far observed the degree of retardation of growth of pneumococci exerted by the whole blood corresponded to the degree of resistance of the corresponding animal to infection by these organisms. The converse, *i.e.* the absence of power in the blood to retard growth, is probably not always a true index of the susceptibility of the animal yielding it to infection. This seems to be true in the case of the cat, particularly on intravenous inoculation.

The whole blood reaction, as pointed out by Heist and his coworkers,[6] makes it possible to detect immunity responses which are not made evident by other methods. Only rabbits were used in the experiments on this point and our conclusions are correspondingly limited. We were unable to detect differences among untreated individuals of the same species. Hence it is improbable that the reaction will enable the detection of pneumonia susceptibility among human individuals, as the Schick test does for diphtheria.

The mechanism of the reaction is of particular interest, especially in connection with the light that its explanation may shed upon the nature of the factors and processes of pneumococcus immunity. Wright[5] designated the phenomenon as the "phagocyto-bactericidal power" of the blood, believing that the phagocytes were concerned in the reaction since plasma did not manifest it; but since defibrinated blood was inactive in this respect, he concluded that the chemical changes associated with the process of coagulation of the blood were essential factors in killing the pneumococci. The present work indicates that opsonization and phagocytosis are the essential agents, since the pneumococcidal reaction does not occur in the absence of either factor. It was shown, moreover, that with either factor constant the reaction varied quantitatively with the quantity of the second factor. It is conceivable that this relation will not be maintained when immune serum exceeds the concentration necessary for complete opsonization of all the organisms, the phagocytic capacity remaining constant. On the other hand, complete opsonization being maintained, there should be no limit to the number of organisms which

an increasing number of phagocytes could hold in check. This relation is similar to that which exists between immune serum and pneumococci in mouse protection experiments. In the latter instance, a certain quantity of antipneumococcus serum (0.2 cc.) protects against a maximum number (0.1 cc. of culture) of organisms, but when the number of organisms is increased, 0.2 cc. or larger quantities of the serum do not protect. This fact indicates that some essential factor (phagocytic capacity?) which is not increased has reached the limit of its power and the reaction fails. To carry the analogy further, the functioning time of the phagocytes in the capillary cultures is limited, and at the expiration of the period of phagocytic activity the reaction ceases. The pneumococci are now free to multiply regardless of the fact that immune bodies are still present. In the mouse protection tests a concentration of immune serum sufficient for complete opsonization continues for a limited time only. If the infection is so heavy that the phagocytes, functioning to the limit of their capacity, cannot destroy all the pneumococci during the opsonization period, some of the organisms escape destruction and are free to grow as soon as the opsonizing factor is eliminated. It has been shown that if the immune serum is kept at a sufficient concentration in the tissues of the infected animals by repeated injections, thus giving the phagocytes a longer working period, all the organisms are destroyed and the animal recovers.[14] This effect is not subject to duplication *in vitro* since the phagocytes which cease functioning because of death cannot be replaced.

It has not been definitely determined whether the retardation of growth of the culture is due merely to an inhibition of multiplication or to an actual destruction of a portion of the organisms, thus prolonging the incubation period. Indications are, however, that the action is one of inhibition only, since none of the cultures became sterile during the course of the experiment. It will be necessary to do further work before the matter is definitely cleared up.

[14] Bull, C. G., *J. Exp. Med.*, 1915, xxii, 466.

SUMMARY.

1. It has been shown that the whole uncoagulated blood of immune animals is not as highly pneumococcidal *in vitro*, as has been claimed by others.

2. Cultures of pneumococci in the fresh whole blood of immune animals, as compared with cultures in the blood of susceptible animals, show a greatly prolonged latent period, and, in a general way, the relative lengths of the latent periods of the cultures correspond to the relative resistances of the animals to infection by these organisms.

3. The blood of animals artificially immunized, both actively and passively, retards the growth of pneumococci in the same manner as the blood of naturally immune animals.

4. Microscopic examination of cultures of pneumococci in immune blood reveals chain formation, growth in clumps, and phagocytosis of the organisms by the polynuclear cells. It also shows that growth occurs first in the free serum, the clot being invaded later.

5. The retardation of multiplication depends on two factors, opsonization of the pneumococci by the immune serum and phagocytosis of the organisms by the polynuclear cells; growth readily occurs when either agent is absent.

6. Pneumococci multiply in defibrinated immune blood because few phagocytes are present after defibrination.

7. Pneumococci grow in the most potent immune blood after mechanical destruction of the white cells.

8. It has not been shown that immune blood does not kill a certain number of the pneumococci with which it is inoculated, but the tentative conclusion has been arrived at that no killing occurs since none of the tests became sterile during the course of our experiments.