STUDIES ON PNEUMOCOCCUS GROWTH INHIBITION.

VII. The Relation of Opsonins to Natural Resistance against Pneumococcus Infection.*

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It seems not improbable that the filling in of some of the many gaps in our knowledge of the mechanism of recovery from bacterial disease and acquired immunity may depend primarily upon a clearer understanding of the processes by which the naturally resistant animal defends itself against pathogenic microorganisms. Information concerning the nature of these processes is as yet largely general in character. Although it has been long recognized that the body is capable of certain well defined antibacterial responses, the exact rôle they play in the destruction of the different species of invading bacteria remains for the most part unknown. The search for immunity factors underlying natural resistance has proved particularly baffling as reactions shown characteristically by the body cells or fluids, following recovery from an infection, have been found to be absent or to occur only irregularly in the naturally immune animal.

A review of the literature on natural immunity to the pneumococcus reveals clearly this lack of a uniform, or even at times detectable, response by the pneumococcus-resistant body. The reaction observed to occur most constantly in studies on this subject is phagocytosis, but while certain workers have found that virulent pneumococci injected into the tissues of pneumococcus-resistant animals were promptly and constantly engulfed by the leucocytes (1, 2), others, apparently employing similar experimental conditions, have noted phagocytosis to be variable (3) or even absent (4). Furthermore,

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doubt has been cast on the assumption that phagocytosis is to be considered the chief, or perhaps even a very important, means of antipneumococcus defense, by the lack of any evidence to show that the serum of resistant animals possesses greater phagocytosis-promoting power or that their leucocytes are more actively phagocytic than the serum and leucocytes of susceptible animals. Indeed highly virulent pneumococci have been found to be uniformly resistant to phagocytosis *in vitro* except in the presence of specific antipneumococcus serum (5–8). Attempts to protect susceptible animals against infection with the serum of highly resistant mammals have not been successful.

The failure to find humoral defense substances in the resistant animal's blood and the inconclusive results of the observations on phagocytosis led investigators to test the action of leucocyte extracts on the pneumococcus, since it seemed certain that the leucocytes must play a rôle in the defense against this organism. It was found that pneumococci seeded into extracts of leucocytes failed to grow and were either reduced in numbers or disappeared entirely (9–11). Furthermore, leucocyte extracts injected during the course of experimental pneumococcus infection were considered to have influenced the course of the disease favorably (12). Pointing toward the primary importance of cellular activity in the antipneumococcus defense mechanism is the further observation that in the pigeon certain of the large fixed tissue cells play an active part in removal by phagocytosis of injected pneumococci (13). The above findings have given rise to the view quite widely held that natural immunity to the pneumococcus, in common with other pathogenic bacteria, depends on certain fundamental characteristics of the body cells and not on circulating antibodies (14–16).

However, of recent years evidence has been accumulating to indicate that natural resistance to pneumococcus infection may depend much more on humoral defense elements than was formerly thought.

As long ago as 1910 Neufeld and Händel (17) found that the blood serum of some normal humans protected mice against otherwise fatal doses of pneumococci. Cecil and Austin (18) showed a protective action in the serum of one out of sixteen normal humans tested. Recently Clough (19) in an extensive study of the protective properties in normal human serum of individuals without antecedent

history of pneumonia,[1] demonstrated definite protective power in 11 of 22 sera tested, against one or more of the three fixed types of pneumococcus. The degree of protective power was usually slight but in five instances the serum protected against from 1000 to 100,000 M.L.D. He failed to find opsonic or agglutinative action in any of these sera. Burhans and Gerstenberger (20) studied the anti-pneumococcal protective power of both mothers' and infants' blood serum and found protective properties in 30 to 40 per cent of more than 100 cases tested. Bull and McKee (21) in 1921 showed that chicken serum conferred on mice well marked protection against many times the lethal dose of highly virulent pneumococci of all four types. They were able to isolate the protective substance from the serum in the water-insoluble globulin fraction. In a study of the mechanism of natural immunity in the dog, Bull (22) found that highly virulent pneumococci injected into the blood stream were agglutinated and carried to the liver and spleen principally. He was unable, however, to demonstrate *in vitro* either agglutinins or opsonins in the dog's serum.

Thus while the findings summarized above show that here and there among the pneumococcus-resistant animal species certain body reactions against the pneumococcus have been detected, they give no indication that there exists a common means of protection, or anti-pneumococcus reactive process, even among the mammals. Our finding, reported in an earlier paper (23), of a constantly demonstrable pneumococcidal action in the blood of the dog and the cat for highly virulent strains which the blood of susceptible animals failed to show, together with Woo's (24) demonstration of a similar action in rabbits' blood against pneumococci of low virulence for the rabbit, suggests that animals naturally immune to pneumococcus infection do possess a common means of defense against this organism. In previous studies no attempt was made to determine the exact nature of the pneumococcus-destroying power of the blood further than to observe that this property did not reside in the serum alone nor in the leucocytes with inactivated serum. It appears to depend on the combined action of the fresh serum and leucocytes. It was the purpose of the present investigation to study the mechanism of this reaction and to determine if possible whether the differences in blood pneumococcidal activity shown by the various animal species depend on differences in the serum constituents or on the character of the leucocytes.

[1] One person gave a history of pneumonia many years previously.

Technique.

The basis of the technique employed in carrying out the phagocytic tests was that originally described by Wright and Douglas (25). It was found necessary, however, to modify this procedure, particularly in regard to two points—the preparation of the leucocytes and the growth phase of the organisms.

Leucocytes.—The leucocytes employed in the following experiments were obtained from aleuronat exudates withdrawn from the pleural cavity 15 to 18 hours after injection of the aleuronat. The solutions used for suspension and washing were the same as those employed in the former work with growth inhibition tests (23). After the second washing in gelatin-salt solution the leucocytes were suspended in gelatin-Locke's solution and were ready for use. A standard suspension was made such that each c.mm. contained 50,000 white blood cells. In order to prevent clumping of the leucocytes it was found necessary to mix red blood cells with them. After the leucocytes had been counted, and before the first centrifugation, 0.75 to 1.5 cc. of a mixture of equal parts of blood and 1 per cent citrate-0.9 per cent salt solution were added to the suspension, the quantity added depending on the richness of the exudate. Centrifugation was carried on at low speed and for as short a time as necessary to completely sediment the corpuscles.

All glassware with which the leucocytes came into contact was cleaned with especial care, according to the technique described in the former publication referred to. The leucocytes were always used fresh and were kept on ice until actually employed in the pipette mixtures.

Organisms.—The Type I pneumococcus employed in Experiments 1 to 5 was a strain originally isolated from a patient with lobar pneumonia and kept for a number of years in blood broth with an occasional animal passage. Immediately before the beginning of the present work it was passed rapidly through ten rabbits and its virulence for rabbits, guinea pigs, dogs and cats tested with the following results: 0.0000001 cc. killed 1500 to 1600 gm. rabbits in 24 to 36 hours; 0.000001 cc. killed guinea pigs of 470 gm. in 15 days; 2.5 cc. to 5 cc. killed dogs weighing from 15 to 20 kilos in 24 to 48 hours; 1 cc. to 5 cc. killed cats of 2000 to 2500 gm. in 48 hours.[2] During the progress of the work this strain was passed through a rabbit approximately once a month in order to eliminate avirulent members which might appear in the culture.

The Pneumococcus Type II used in Experiment 6 was a strain secured from The Rockefeller Institute, originally isolated from a case of lobar pneumonia and designated as D 39. Even after passage through a series of rabbits, its virulence for this animal remained low—0.1 to 0.01 cc. was required to kill rabbits of 1500 to 1800 gm.

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[2] This was the most virulent organism we had in our possession. Other strains of Types II, III and IV were much less virulent and hence less suitable for the purposes of this work.

The growth phase, media employed and suspension of pneumococci for use will be dealt with in the first two experiments.

Sensitization.—After thorough mixture of pneumococcus suspension with serum in a 15 cc. centrifuge tube, sensitization was carried on in the water bath at 37°C. for 1 hour unless otherwise stated. The organisms were then sedimented by centrifugation at high speed for 1 hour, the serum next completely removed and the pneumococci taken up in sufficient Locke's solution pH 7.6 to make a suspension somewhat more concentrated than that originally added.

Opsonic Test.—Wright's capillary pipettes and technique of making mixtures were used. The pipettes were sealed with paraffin and incubated for 45 minutes. After opening, the supernatant fluid was expelled gently and only a small amount used to wash out the sedimented cells. In this way a thick cell suspension was obtained. Films were made on cover-slips 22 × 35 mm. and stained with Cross's stain. To estimate the degree of phagocytosis, 100 leucocytes were counted in each preparation. The per cent of leucocytes taking part in phagocytosis, as well as the number of leucocytes containing 5 or more pairs of pneumococci, were recorded. In some experiments counts of the per cent of leucocytes containing 20 or more pairs were made. Wright's phagocytic index was not determined. In order to estimate the variation in counts from pipette to pipette, and in different preparations from the same pipette, counts were made on a number of pipettes containing the same mixture of serum, leucocytes and pneumococci. The amount of variation in the counts thus made was found to be conditioned chiefly by the degree of phagocytosis occurring. With a mixture showing marked phagocytosis, the counts from pipette to pipette were surprisingly uniform —the variation was usually not more than 5 to 10 per cent. But as the intensity of phagocytosis lessened, the differences between pipettes increased until fluctuations of 25 to 50 per cent above or below might be expected with mixtures yielding only slight phagocytosis. Relatively little difference was found between counts on duplicate preparations from the same pipette. Spontaneous phagocytosis of unsensitized pneumococci was practically absent.

EXPERIMENTAL.

Concentration of Normal Antipneumococcus Opsonins in Dog Serum.

Since it seemed most probable that the pneumococcidal power of the blood of naturally resistant animals was to be explained by phagocytosis and intracellular digestion, investigation of the opsonic properties of the serum and phagocytic activities of the leucocytes was undertaken first. The consistently negative results of previous investigators indicated clearly, however, that no further information could be gained by carrying out phagocytic tests in the usual way. A clue to the correct method of approach was afforded by the quanti-

tative nature of the pneumococcus-destroying action shown by the serum-leucocyte mixtures. Given a fixed amount of serum, the number of pneumococci killed never exceeded a definite maximum amount of standard pneumococcus suspension, which was quite small in comparison with the quantity of serum used. The number of leucocytes could be varied widely without influencing the result. This made it seem likely that if opsonic action played a part in the process, it could be demonstrated only by employing a relatively large ratio of serum to pneumococci. In the following experiment a fixed quantity of pneumococcus suspension was sensitized with varying amounts of dog serum and the resulting degree of phagocytosis by dog leucocytes observed.

TABLE I.

Concentration of Normal Opsonins in Dog Serum.

Dog-serum-sensitized pneumococci + dog leucocytes + dog serum 1:5 dilution.

Quantity of dog serum used for sensitization	Amount of pneumococcus suspension	Degree of phagocytosis	
		Per cent of leucocytes taking part in phagocytosis	Per cent of leucocytes containing 5 or more pairs
cc.	*cc.*		
5.0	0.1	99	96
2.5	"	87	62
1.0	"	45	12
0.1	"	0	0
Control with unsensitized pneumococci.....		0	0

Experiment 1.—(Table I.) A normal dog weighing 16 kilos was bled for serum just before injection of aleuronat on day preceding test. The serum was kept in the ice box overnight. The organisms, cultured in 1 per cent rabbit serum broth, and having passed through the phase of active growth, were freed from their culture fluid by centrifugation, then suspended in gelatin-Locke's solution pH 7.8. The density of the suspension was approximately that of the standard suspension used in former experiments (23). Actual standardization was not made. The pneumococci sedimented from the sensitizing serum showed well marked clumping in all but the tube containing the smallest quantity of serum. The agglutinated mass could be broken up fairly easily with a capillary pipette in the gelatin-Locke's solution. However, with the largest amount of serum small clumps still remained. The phagocyting cells were almost entirely polymorphonuclears. Only an occasional active monocyte seen.

The results of the above experiment are shown in Table I. It was found that with a ratio of sensitizing serum to pneumococcus suspension of 50 to 1, marked phagocytosis occurred. Practically all the leucocytes were packed with organisms. Diminishing the quantity of sensitizing serum resulted in a progressive decrease of phagocytosis until, with equal parts of serum and pneumococcus suspension, phagocytosis no longer occurred. A comparison of the figures under per cent of leucocytes containing five or more pairs gives a more accurate idea of the relative degree of phagocytosis than do the percentages in the first column. Repetitions of this experiment with both dog and cat serum under identical conditions gave essentially the same results.

The Effects of Growth Phase and Culture Fluid on Opsonic Action.

In further experiments on the opsonic action of dog and cat serum, marked variations in the degree of resulting phagocytosis were noted from test to test. These variations appeared to be greater than could be accounted for by individual differences in the animals providing the serum. It was then observed in several experiments in which the degree of phagocytosis was relatively low that the pneumococci used were in the active growth phase and continued to grow in the sensitizing serum. This led to a consideration of the effect of growth state an opsonization and phagocytosis. In the preceding paper one of us (26) showed that the soluble specific substance of the pneumococcus, both in purified form and in the filtrate of actively growing cultures, could inhibit to a marked degree the pneumococcidal action of normal serum-leucocyte mixtures. The nature of this inhibiting effect was not investigated at that time but it was considered most probably to be an interference with opsonization analogous to the action of Rosenow's "virulin" (27). In spite of the fact that the organisms used in the present experiments had been separated from their growth products before use, it seemed not improbable that an actively growing highly virulent pneumococcus might elaborate, during the period of suspension in the sensitizing serum, sufficient soluble substance to protect itself partially or completely against the action of normal opsonins. The possibility also existed that the growth products of pneumococci might affect the leucocytes so as to diminish

their phagocytic activity, although the result of previous work made this seem unlikely. The next experiment was devised to test the effect of growth phase and soluble substance on the phagocytic activity of normal dog serum and leucocytes.

TABLE II.

Effect of Growth Phase and Culture Fluid on Opsonic Activity of Dog Serum.

Dog-serum-sensitized pneumococci + dog leucocytes + dog serum 1:5 dilution.

Growth phase	Amount of sensitizing serum	Fluid added to sensitizing serum	Time of sensitization	Fluid added to pipette mixtures	Degree of phagocytosis		
					Per cent of leucocytes taking part in phagocytosis	Per cent of leucocytes containing 5 or more pairs	Per cent of packed cells; leucocytes containing 20 or more pairs
	cc.						
Lag	5	0.4 cc. broth	1 hr.	—	96	91	45
Active	"	" " "	" "	—	53	10	0
Lag	"	" " "	30 min.	—	98	83	6
Active	"	" " "	" "	—	87	53	2
Lag	"	" " "	15 min.	—	90	58	10
Active	"	" " "	" "	—	76	49	1
Lag	"	" " "	1 hr.	Culture fluid equal parts	78	76	34
Lag	"	0.4 cc. culture fluid	" "	—	62	34	8
	Controls with unsensitized pneumococci						
Lag				—	1	0	0
Active				—	1	0	0

Experiment 2.—(Table II.) Serum and leucocytes secured as in Experiment 1 from a normal dog weighing 17 kilos. To obtain pneumococci suitable for studying the lag state, seedings were made into flasks containing meat infusion broth to a depth of 1.5 cm.[3] The growth phase was tested by replanting a small quantity

[3] The employment of a relatively large surface area of culture fluid for the purpose of constant and early cessation of active growth was suggested by Avery's work on the occurrence of peroxide in pneumococcus cultures (Avery, O. T., and Morgan, H. J., *J. Exp. Med.*, 1924, xxxix, 275). A small quantity of dextrose, 0.05 per cent, was added to the broth on account of the very low sugar content of broth made from North China beef.

of the culture, freed from its fluid, into 50 per cent rabbit serum broth. Growth was easily detectable macroscopically by comparison with a control suspension kept in the ice box. It was found that 0.05 cc. of an actively growing culture seeded into 33 cc. of broth contained in a 100 cc. Erlenmeyer flask, which gave the desired depth of fluid, usually completed its active growth after 16 to 18 hours incubation. Plates of dilutions of these cultures showed that for a period of 2 to 3 hours after growth had ceased there was no appreciable diminution in the number of colonies. Several flasks were seeded the day before the test at times appropriate to give cultures of 14, 16, 18 and 20 hours. The youngest culture showing no growth in the rabbit serum broth at the end of 2 hours was chosen for the test.

The actively growing culture used was a 14 hour test-tube growth. The supernatant fluid of this culture, centrifuged until microscopically free from pneumococci, was employed in the experiment. Suspensions of the organisms were made as in the preceding experiment.

The growth phase of the organism was found to exert a marked effect on the degree of phagocytosis of pneumococci by dog serum and leucocytes (Table II). Pneumococci in the state of lag during sensitization were subsequently engulfed to a much greater extent than were the organisms which continued to grow actively during this period. The contrast between active growth and lag phase is shown most strikingly by the pneumococci sensitized for 1 hour, which gave the maximum amount of phagocytosis of the lag organisms but resulted in only a very moderate degree of ingestion of the actively growing pneumococci. The effect of growth during sensitization is further shown by a comparison of the phagocytosis percentages of actively growing organisms sensitized for varying periods of time. An hour's sensitization gave considerably less phagocytosis than did the 15 and 30 minute periods.

The addition to the sensitizing serum of a small quantity of the supernatant fluid of the actively growing culture reduced considerably its opsonic action on pneumococci in the lag phase. (Compare Pipettes 1 and 8, Table II.) That the pneumococcus soluble substance acts primarily on the opsonins and not on the leucocytes is indicated by the fact that culture fluid mixed with leucocytes and previously sensitized organisms did not retard to any extent the resulting phagocytosis. The difference between Pipettes 1 and 7 can be accounted for by the increased amount of fluid in Pipette 7, which

always lowers the phagocytosis percentage a little. In further experiments it was found that by progressively increasing the amounts of culture fluid in the sensitizing serum, opsonic action could be almost completely inhibited. Broth in equivalent amounts produced no such effect.[4] This result is quite in accord with Rosenow's findings.

Phagocytosis of Pneumococci Sensitized by Alien Serum.

Having determined those factors which influence phagocytosis so markedly in homologous serum-leucocyte mixtures, the next step was

TABLE III.

Comparative Opsonic and Phagocytic Activity of Serum and Leucocytes of Dog and Rabbit.

Sensitized pneumococcci + leucocytes + serum diluted 1:5.

Kind and quantity of sensitizing serum	Amount of pneumococcus suspension	Leucocytes	Serum diluted 1:5	Degree of phagocytosis	
				Per cent of leucocytes taking part in phagocytosis	Per cent of leucocytes containing 5 or more pairs
	cc.				
Dog 5 cc.	0.1	Dog	Dog	98	94
" " "	"	Rabbit	Rabbit	69	45
Rabbit 5 cc.	"	Dog	Dog	0	0
" " "	"	Rabbit	Rabbit	1	0
Dog 5 cc. inactivated at	"	Dog	Dog	17	1
56°C.	"	Rabbit	Rabbit	1	0
Controls with unsensitized pneumococci		Dog	Dog	0	0
		Rabbit	Rabbit	0	0

to ascertain whether the opsonins of the resistant animals sensitize the pneumococcus for phagocytosis generally, as do immune opsonins, or whether they are effective only with their own leucocytes. One of the chief difficulties met with in carrying out such tests lies in the injurious action of alien serum on the leucocytes. Hence in the following experiment in which pneumococci were sensitized with dog

[4] The effect of pneumococcus soluble substance on opsonic action will be taken up in detail in a further communication.

serum and tested with rabbit leucocytes, special precautions were taken to remove the serum as completely as possible from the sensitized organisms.

Experiment 3.—(Table III.) Serum and leucocytes from a dog weighing 24 kilos. Rabbit serum from a rabbit weighing 1650 gm. Rabbit leucocytes from another animal of the same weight. Dog serum inactivated by heating at 56°C. for 30 minutes. Pneumococci, for sensitization in the lag phase, were prepared as in the preceding experiment. After centrifugation the last portion of the supernatant serum was removed with a fine capillary pipette. Next, the surface of the sediment and the lower 2 to 3 cm. of the walls of the centrifuge tube were washed with a small quantity of gelatin-Locke's solution pH 7.4 run in slowly with the tube tilted almost horizontal. This fluid was removed with a capillary pipette and more gelatin-Locke's added for suspension. The dog-serum-sensitized pneumococci showed moderate clumping. No agglutination of the organisms suspended in the rabbit serum was seen. No hemolysis or clumping of rabbit cells was observed in the capillary pipette mixtures. Slight hemolysis and methemoglobin formation occurred in all the pipettes containing dog cells.

Virulent pneumococci sensitized by dog serum as in the above experiment were engulfed by rabbit leucocytes to a marked degree (Table III). Many of the leucocytes were packed with organisms. In striking contrast is the absence of phagocytosis of rabbit-serum-treated pneumococci by either dog or rabbit leucocytes. The failure of dog leucocytes to ingest unsensitized organisms would indicate that the behavior of these cells in the presence of unaltered virulent pneumococci does not differ essentially from that of rabbit leucocytes. The greater activity shown by dog leucocytes for the dog-serum-sensitized pneumococci may be accounted for by a certain degree of cytotoxic action of dog serum on the rabbit leucocytes. In other similar experiments analogous results have been obtained. Tests of the comparative phagocytic activity of dog and rabbit leucocytes in the presence of specific antipneumococcus horse serum, 1:200 dilution, have shown rabbit leucocytes to be fully as active as dog leucocytes. Inactivation of the dog serum largely destroyed its opsonic activity.

The growth state of the pneumococcus was found to exert a more pronounced effect on heterologous than on homologous serum-leucocyte systems. Rabbit leucocytes showed relatively little phagocytic activity for actively growing pneumococci sensitized by dog serum. That the greater phagocytability of these organisms when sensitized

during the lag phase, was not due to impaired vitality was determined by rabbit virulence tests which showed them to be quite as virulent as the actively growing pneumococci.

Occurrence of Opsonic Activity in Sera of Cat, Sheep, Pig and Horse.

Tests were next undertaken to determine whether the serum of other animals naturally resistant to the pneumococcus likewise possessed opsonins for this organism, and whether the difference in phagocytic activity between their serum and leucocytes and those of susceptible animals lay also in the humoral rather than in the cellular elements of the blood. For this purpose the cat, sheep, pig and horse were chosen to represent a wide variety of the resistant animals, while the human, rabbit and guinea pig were employed as typically susceptible ones.

Experiment 4.—(Table IV.) The data assembled in Table IV represent three separate experiments. The cat, sheep and rabbit elements were tested at one time, the pig, horse, human and rabbit at another, and the pig, guinea pig and rabbit at still a third. The animals employed were large, normal and full grown adults. The human serum was obtained from two normal individuals. Agglutination was present in all the tubes with sera of resistant animals, but none occurred in the sera of susceptible animals. No hemolysis was observed in the cross phagocytic tests. Predominantly active cells were polymorphonuclears.

The uniform occurrence in the serum of all the pneumococcus-resistant animals tested of opsonic activity that can be demonstrated not only with its own but with leucocytes of alien species, both resistant and susceptible, is strikingly shown in Table IV. The lack of phagocytic activity on the part of the leucocytes of resistant animals, for pneumococci exposed to the action of susceptible animal's serum, is equally noteworthy. The varying degrees of phagocytosis shown by the serum and leucocytes of the different animals are not to be taken as strictly comparative of the opsonic content of their sera even when tested at the same time against the same alien leucocytes because of certain variable factors which cannot be readily controlled, such as intensity of agglutination and cytotoxic action. However, the purpose of the present work, namely, to determine the differences between the serum and leucocytes of resistant and susceptible animals with respect to their opsonic and phagocytic activities is fulfilled by

the conditions of the above experiments. Numerous repetitions of different parts of Experiment 4 gave essentially the same results.

TABLE IV.

Opsonic Action of Serum of Resistant and Susceptible Animals.

Sensitized pneumococci + leucocytes + serum diluted 1:5.

	Kind and quantity of sensitizing serum	Amount of pneumococcus suspension	Leucocytes	Serum diluted 1:5	Degree of phagocytosis	
					Per cent of leucocytes taking part in phagocytosis	Per cent of leucocytes containing 5 or more pairs
		cc.				
Resistant animals	Cat 10 cc.	0.1	Cat	Cat	96	91
	" " "	"	Rabbit	Rabbit	56	29
	Sheep 10 cc.	"	Cat	Cat	89	74
	" " "	"	Rabbit	Rabbit	66	44
	Pig 10 cc.	"	Pig	Pig	91	86
	" " "	"	Dog	Dog	53	33
	" " "	"	Rabbit	Rabbit	83	73
	" " "	"	Guinea pig	Guinea pig	96	88
	Horse 10 cc.	"	Rabbit	Rabbit	27	19*
Susceptible animals	Rabbit 10 cc.	"	Rabbit	Rabbit	0	0
	" " "	"	Sheep	Sheep	0	0
	" " "	"	Cat	Cat	0	0
	Guinea pig 10 cc.	"	Guinea pig	Guinea pig	11	0
	" " " "	"	Pig	Pig	5	0
	" " " "	"	Dog	Dog	1	0
	Human 10 cc.	"	Rabbit	Rabbit	0	0
Controls with unsensitized pneumococci			Cat	Cat	0	0
			Sheep	Sheep	0	0
			Pig	Pig	2	0
			Dog	Dog	1	0
			Guinea pig	Guinea pig	0	0
			Rabbit	Rabbit	0	0

* Agglutination massive and could be only partially broken up.

A study was made also of the opsonic activity of the blood serum of birds. As this part of the work is less complete than that on mam-

mals, a detailed report is reserved for a future communication. Suffice it to say here that well marked opsonic action was demonstrated with the homologous serum and leucocytes of the chicken and pigeon although not as pronounced as that shown by mammalian blood. Tests with alien blood elements failed, except in one or two instances, to show any degree of phagocytosis.

Significance of Normal Antipneumococcus Opsonins.

Experiments were next undertaken with the purpose of ascertaining as far as possible the importance of opsonic action in the pneumococcus-destroying processes of the body. In previous studies on the pneumococcidal action of serum-leucocyte mixtures of the dog and the cat it was found that inactivation of the serum at 56°C. deprived the mixture of its pneumococcus-killing action. The further finding in the present work that heating to 56°C. largely destroys the opsonic properties of the normal serum suggests that pneumococcidal power of the blood is dependent on the opsonic activity of the serum. To test this presumption a series of experiments was performed with the serum and leucocytes of the dog, cat, sheep and pig, comparing opsonic action with pneumococcidal potency. A specimen protocol is shown in Table V.

Experiment 5.—(Table V.) Serum and leucocytes obtained from a normal full grown pig. The growth inhibition tests were carried out as detailed in previous papers (23, 28). The opsonic tests were performed as in the preceding experiments. The sedimented organisms from both the inactivated and active serum tubes showed agglutination which was most marked with the fresh serum, least with the 65°C. heated serum. Leucocyte extracts were prepared in two ways: (1) by shaking washed leucocytes suspended in Locke's solution with glass beads for 2 hours, at the end of which time only nuclear remains and stroma were seen; and (2) by grinding leucocytes in very fine sand for 20 minutes, which resulted in their complete maceration.[5] These suspensions were then centrifuged at high speed and the clear supernatant fluid used in the test.

The pronounced pneumococcidal action shown by the serum-leucocyte mixtures of all the animals tested was entirely abolished

[5] Leucocyte extracts were prepared as well in the experiments with dog, cat and sheep serum-leucocyte mixtures by freezing and thawing and also extracting for 24 hours with distilled water.

when heated serum was substituted for fresh serum. Comparative opsonic tests on the heated and unheated sera showed that the former

TABLE V.

Opsonic Activity and Pneumococcidal Power of Pig Serum and Leucocytes: Action of Leucocyte Extract and Effect of Serum Inactivation.

A. Growth Inhibition Test.

Pig serum 0.3 cc. + pig leucocytes or extract 0.1 cc. + pneumococcus suspension 0.1 cc.

	Amount of standard suspension	Growth as shown by color changes* at hrs.			Survival of pneumococci at 72 hrs.	
		17	42	72	Stained film	Culture
	cc.					
Fresh serum	0.01	0	0	0	0	0
	0.001	0	0	+	+	+
	0.0001	0	0	0	0	0
	0.00001	0	0	0	0	0
	0.000001	0	0	0	0	0
Serum inactivated at 56°C.	0.0001	+	++++		+	
	0.00001	0	++++		+	
	0.000001	0	++	++++	+	
	0.0000001	0	+++	++++	+	
Serum inactivated at 65°C.	0.0001	++++			+	
	0.00001	++++			+	
	0.000001	++++			+	
	0.0000001	++++			+	
Leucocyte extract (shaken)	0.0001				+	
	0.00001				+	
	0.000001				+	
	0.0000001				+	
Leucocyte extract (ground)	0.0001				+	
	0.00001				+	
	0.000001				+	
	0.0000001				+	
Controls with fresh serum only	0.000001				+	
	0.0000001				+	

* Degrees of methemoglobin formation.

TABLE V—*Concluded.*

B. Opsonic Test.

Pig serum-sensitized pneumococci + pig leucocytes + pig serum 1:5 dilution.

Quantity of sensitizing serum	State of sensitizing serum	Amount of pneumococcus suspension	Degree of phagocytosis		
			Per cent of leucocytes taking part in phagocytosis	Per cent of leucocytes containing 5 or more pairs	Per cent of packed cells; leucocytes containing 20 or more pairs
cc.		*cc.*			
5	Fresh	0.1	93	87	25
"	Inactivated at 56° C.	"	57	32	3
"	Inactivated at 65° C.	"	35	9	0
Control with unsensitized pneumococci....			2	0	0

had lost much of their opsonic potency but still retained varying degrees of this property. The opsonins of the pig were found to be the most resistant to heating. As indicated in Table V pig serum heated to 56°C. for half an hour showed well marked opsonic activity, though considerably less than that of the fresh serum. This finding in association with the retardation of growth produced by the 56° heated serum and leucocytes suggests the possibility that while the heated-serum opsonins can still cause phagocytosis, the sensitization produced by opsonins thus injured is inadequate for complete intracellular digestion.[6] Heating the sera of the dog and the cat to 56°C. destroyed their opsonic properties almost completely and there was no growth retardation in the growth inhibition tests with the heated serum and leucocytes of these animals. Sheep serum opsonins were more resistant though not as much so as those of the pig. That part of the experiments dealing with the effect of leucocyte extracts will be taken up later.

[6] That the phagocytosis observed was not due to reactivation of the heated opsonins by the small amount of fresh serum present in the pipette mixture, was determined by comparing the effect of adding to heated serum-sensitized pneumococci and leucocytes, heated serum, fresh serum and no serum. The resulting degree of phagocytosis in all three mixtures was practically identical.

The results of the foregoing experiments, which were repeated many times with substantially the same findings, indicate that opsonins must play a very important, if not decisive, rôle in the pneumococcidal action of serum-leucocyte mixtures. In the absence of opsonins the leucocytes of pneumococcus-resistant animals apparently do not possess any greater ability either to engulf or to cause destruction of virulent pneumococci than do the leucocytes of susceptible species. There still remained the possibility, however, that the leucocytes of the former type of animal may possess within their cell substance pneumococcus-destroying principles which are not present in the cells of the latter. Experiments were accordingly devised in an attempt to determine whether such differences could be demonstrated in the leucocytes of the several animal species studied above.

First, observations were made on the rate of intracellular digestion of dog and cat serum-sensitized pneumococci by dog, cat and rabbit leucocytes. Capillary pipette mixtures were examined microscopically after intervals of 1 to 6 hours incubation. The rabbit leucocytes appeared to digest the engulfed pneumococci fully as effectively as those of the dog and cat. More conclusive information as to the ultimate fate of the ingested organisms was sought by adding pneumococci sensitized by dog serum,[7] both in the active and lag phase, to rabbit serum-leucocyte mixtures, which were then tested for growth-inhibitory effect. The rabbit leucocytes failed to show any pneumococcidal action. This experiment was repeated with sheep serum from which the anti-rabbit cytotoxins had been removed by successive additions of rabbit blood cells at 5°C. The result was the same. Likewise, rabbits injected with small quantities of dog and cat serum-sensitized pneumococci, both with and without the removal of the sensitizing serum, invariably succumbed to pneumococcus infection. However, the results of these tests are unsatisfactory from the standpoint of determining the pneumococcus-destroying powers of the leucocytes because cytotoxic action of the alien serum cannot be completely eliminated.

The action of leucocyte extracts on pneumococci was then tested, as shown in Table V. Extracts of leucocytes from the dog, cat, sheep and pig were found to be without pneumococcidal properties. The explanation of the discrepancy between our results and those of former workers may well be found in the reaction of the extracts used. Rous (29) has recently shown that the protoplasm of the leucocyte is distinctly acid. In our work the use of adjusted and buffered solu-

[7] These organisms were mixed with serum in sealed tubes and agitated during incubation so as to secure complete sensitization of all the pneumococci. The serum was completely removed after centrifugation.

tions and the addition of a relatively large quantity of serum to the leucocyte extract probably acted to maintain its reaction at about that of blood. The extracts

TABLE VI.

Comparative Pneumococcidal and Opsonic Activity of Adult Rabbit and Young Hare Serum and Leucocytes.

A. Growth Inhibition Test.

Serum 0.3 cc. + leucocyte suspension 0.1 cc. + pneumococcus suspension 0.1 cc.

Serum	Leucocytes	Pneumococcus suspension	Growth as shown by color changes at hrs.			Survival of pneumococci at 72 hrs.	
			16	48	72	Stained film	Culture
		cc.					
Adult rabbit	Adult rabbit	0.01	+++	++++		+	
		0.001	0	++	++++	+	
		0.0001	0	0	0	0	0
		0.00001	0	0	0	0	0
		0.000001	0	0	0	0	0
		0.0000001	0	0	0	0	0
	Young hare	0.01	++	++++		+	
		0.001	0	++	++++	+	
		0.0001	0	0	0	0	0
		0.00001	0	0	0	0	0
		0.000001	0	0	0	0	0
		0.0000001	0	0	0	0	0
Young hare	Adult rabbit	0.01	+++	++++		+	
		0.001	+++	++++		+	
		0.0001	+	++++		+	
		0.00001	0	++++		+	
		0.000001	0	++++		+	
		0.0000001	0	++++		+	
	Young hare	0.01	++++			+	
		0.001	+++	++++		+	
		0.0001	++	++++		+	
		0.00001	++	++++		+	
		0.000001	+	++++		+	
		0.0000001	0	++++		+	
Controls with serum only							
Adult rabbit		0.0000001				+	
Young hare		0.0000001				+	

TABLE VI—*Concluded.*

B. Opsonic Test.

Sensitized pneumococci + leucocytes + serum diluted 1:5.

Kind and quantity of sensitizing serum	Amount of pneumococcus suspension	Leucocytes	Serum diluted 1:5	Degree of phagocytosis	
				Per cent of leucocytes taking part in phagocytosis	Per cent of leucocytes containing 5 or more pairs
	cc.				
Adult rabbit 5 cc.	0.05	Adult rabbit	Adult rabbit	65	48
" " " "	"	Young hare	Young hare	60	47
Young hare 5 cc.	0.05	Adult rabbit	Adult rabbit	0	0
" " " "	"	Young hare	Young hare	4	1
Controls with unsensitized pneumococci		Adult rabbit	Adult rabbit	0	0
		Young hare	Young hare	0	0

employed by previous investigators may have been acid and hence acted as unfavorable media for the viability of pneumococci. Leucocyte extracts prepared with unbuffered neutral solutions were indeed found to have a slightly higher H ion concentration than the original solution used.

Although largely negative in character, the above findings contribute further evidence in support of the probability that the different animal leucocytes behave in a like manner toward pneumococci. Much more direct evidence of the determining influence of opsonic action in intracellular digestion and natural resistance was obtained from a comparative study of the sera and leucocytes of adult and young rabbits or hares. As was shown in a previous communication (24), the relative resistance of full grown rabbits against certain strains of pneumococcus is associated with the presence of pneumococcidal properties in their blood which are entirely lacking in the highly susceptible young animal. It was found possible to analyze the nature of this difference in blood characteristics within the species by the use of methods that had failed in attempts with alien serum and leucocytes.

Experiment 6.—(Table VI.) Adult rabbit serum and leucocytes obtained from a white male rabbit, weight 1500 gm. The young hare leucocytes were secured from a young Belgian hare, weight 520 gm., and the serum from this same hare and another weighing 490 gm. The growth inhibition test was carried out as before. The organism employed was a Type II pneumococcus, which possessed but little virulence for adult rabbits or hares. 0.1 to 0.01 cc. was required to kill the full grown animal, but 0.000001 cc. killed 500 to 600 gm. Belgian hares in 2 to 3 days. For the phagocytic test, pneumococci in the active growth phase were used and sensitization was carried out for 30 minutes. The test was conducted otherwise as previously. The pneumococci sedimented from the adult rabbit serum showed agglutination, those from the young rabbit serum were unclumped.

The results of the experiment are shown in Table VI. A serum-leucocyte mixture consisting of adult rabbit serum and either adult rabbit or young hare leucocytes was found to possess marked growth-inhibitory and pneumococcus-destroying properties. On the other hand, mixtures composed of young hare serum and adult rabbit or young hare leucocytes were entirely lacking in this action. This difference in the effectiveness of the two kinds of serum is to be accounted for by the finding that the large rabbit serum showed well marked opsonic activity both with its own and the young hare leucocytes, while in the serum of the young hare no opsonins could be demonstrated. In these two animals of widely varying susceptibility to pneumococcus infection it seems evident that resistance is dependent upon the presence of opsonins in the serum, since the leucocytes of the young hare are quite as effective in digesting adult serum-sensitized pneumococci as are the leucocytes of the full grown, highly refractory animal.

It is to be noted that actively growing organisms were used in the opsonic test. With Type II pneumococci in the lag phase, agglutination was so massive and tenacious as to interfere markedly with phagocytosis.

Normal Antipneumococcus Agglutinins.

As recorded in the preceding protocols, agglutination occurred wherever opsonic action was detected. Experimental conditions found to be optimum for the demonstration of opsonic activity likewise brought out the most pronounced agglutination. Within certain limits the degree of agglutination observed paralleled the extent to

which the sensitized pneumococci were phagocyted. This parallelism ceased when the intensity of the agglutination reached the point where the clumps were so large that they could no longer be broken up. Heating the serum caused a diminution in both its agglutinative and opsonic properties to, roughly, the same degree. That this clumping of the pneumococci sedimented from the serum of pneumococcus-resistant animals was a true agglutination is shown by the fact that the clumps could never be completely comminuted even after prolonged mixing with a fine capillary pipette. While the suspension might have a smooth appearance (it usually retained a finely granular character), agglutinated masses of pneumococci were always observed microscopically except in instances where the macroscopic clumping had been very slight.

It is not improbable that the packing of the organisms by centrifugation at high speed had much to do with the successful demonstration of these normal serum agglutinins. Gates showed that the agglutination of meningococci could be greatly hastened and intensified by centrifuging (30).

DISCUSSION.

The results of the foregoing experiments strongly suggest that the differences in resistance which certain mammals normally exhibit toward pneumococcus infection are to be accounted for chiefly by the concentration of antipneumococcus opsonins present in their blood. While the markedly resistant animals possess a relatively high concentration of these immune substances (or serum state), the blood of susceptible ones is so poor in this property that it cannot deal with the highly virulent pneumococci but still has a sufficient concentration to sensitize and thereby make possible the destruction of pneumococci of low virulence for the species.

It is realized that the data presented do not entirely exclude the existence of blood cellular differences between resistant and susceptible animals, but the marked phagocytic and intracellular digestive activity shown by the leucocytes of susceptible animals for pneumococci sensitized by the serum of resistant species, makes it seem probable that no striking variations in this function obtain among the several kinds of leucocytes studied. In support of this

inference is the previous finding that the addition of a small quantity of specific immune serum to the serum-leucocyte mixture of the rabbit confers on it marked lethal power for highly virulent pneumococci (31), thus showing that the leucocytes of a susceptible animal are capable of killing such organisms when adequately sensitized.

The degree and type of pneumococcidal action which can be demonstrated in the resistant mammal's blood, together with the cellular picture of the pneumococcus lesion, indicates that in these animals the polymorphonuclear leucocytes are principally responsible for the destruction of pneumococci. Phagocytosis by the fixed tissue cells of the reticulo-endothelial system may, however, play a contributory rôle. The possibility that the body possesses also a mechanism for the extracellular dissolution of pneumococci is by no means excluded even though no conclusive evidence for the existence of such a process has been thus far found.

The general parallelism found to exist between the agglutinative and opsonic properties of the serum of pneumococcus-resistant animals provides some additional evidence in favor of the view held by many that these two reactions are different manifestations of a single immune process or substance.

SUMMARY.

A study was made of the pneumococcidal action of serum-leucocyte mixtures of pneumococcus-resistant animals with a view to determining whether this property of the blood is to be accounted for by the presence of certain serum constituents or by cellular characteristics which are lacking in the blood of susceptible animals. By means of a method specially developed for this purpose, it was found that, after adequate contact with the serum of pneumococcus-resistant animals, virulent pneumococci were phagocyted actively not only by the homologous leucocytes but also by the leucocytes of other resistant and susceptible animals. On the other hand, pneumococci exposed to the action of the serum of pneumococcus-susceptible animals were not taken up by the leucocytes of either the resistant or susceptible species. All the resistant animals tested, dog, cat, sheep, pig and horse, showed marked opsonic properties in their blood serum which were not found in the serum of susceptible ones, rabbit, guinea pig

and human. There appeared, however, to be no essential difference in the phagocytic activity of the leucocytes from the various animals.

It was then shown that the pneumococcus-destroying power of serum-leucocyte mixtures was entirely abolished when heated serum was substituted for fresh serum and that such heated serum had lost much of its opsonic potency. Neither the living leucocytes alone nor extracts of the leucocytes were observed to exert any killing action on pneumococci. Further evidence of the controlling influence of opsonic action in the antipneumococcus defence mechanism of the blood, and its importance in natural resistance, was afforded by a study of the opsonin content and leucocytic functions of the blood of full grown and young rabbits as related to their widely varying degrees of pneumococcus susceptibility.

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