ON THE NATURE OF BACTEREMIA IN EXPERIMENTAL PNEUMOCOCCAL PNEUMONIA IN THE DOG

II. DISAPPEARANCE OF PNEUMOCOCCI FROM THE CIRCULATION IN RELATION TO THE BACTERICIDAL ACTION OF THE BLOOD IN VITRO

BY LUCIEN A. GREGG,* M.D., AND O. H. ROBERTSON,‡ M.D.

(*From the Department of Medicine and the Douglas Smith Foundation for Medical Research of the University of Chicago, Chicago*)

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In the preceding study of the relationship of the natural pneumococcidal-promoting activity of the serum to bacteremia in dogs with experimental pneumococcal pneumonia (1), it was found that the blood of non-bacteremic dogs retains its natural humoral immunity with a remarkable degree of constancy. The occurrence of bacteremia was followed by wide individual variations in the concentration of these antipneumococcal properties, ranging from complete disappearance in the presence of relatively few circulating pneumococci to their persistence in blood containing great numbers of microorganisms. These fluctuations were interpreted to signify differences in the body's capacity to elaborate new antibodies in response to the continued escape of pneumococci from the pulmonary lesion. That the observed variation in pneumococcal immune substances represented corresponding degrees of pneumococcidal power of the blood seemed evident from our tests performed with the shed blood; but to what degrees this action of the blood *in vitro* reflects the ability of the body to deal with pneumococci in the circulation, our observations failed to elucidate, nor did they provide any data as to whether the pneumococcus-clearing action of the blood is more or less effective in the diseased animal than in the normal one.

It was felt that a profitable approach to these questions could be made by observing the effects of artificially induced pneumococcemia in normal and diseased (pneumonic) animals and that such information would contribute to the solution of certain other fundamental problems presented by the bacteremic state, such as the rate of escape of pneumococci from pulmonary lesions, the capacity of the blood stream to dispose of them, and the mechanism by which the body controls the degree of blood invasion in the absence of demonstrable humoral immunity.

* Present address: Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh.

‡ Present address: Department of Biological Sciences, Stanford University, Stanford.

The techniques and procedures employed were the same as those described in the previous paper (1).

EXPERIMENTAL

Clearing of Intravenously Injected Pneumococci from the Blood Stream of Normal Dogs

Attempt was made first to measure the rate at which injected pneumococci disappear from the blood of the normal dog.

Experiment 1 (Table I).—Five normal dogs weighing from 9 to 13 kilos were injected intravenously with a suspension of approximately 1 billion pneumococci obtained from 1 cc. of culture. Blood cultures were made at 2, 5, 15, and 30 minutes after the injection and then at longer intervals.

One dog was sacrificed at the end of the first 30 minutes of the experiment. Two were reinoculated 2 hours after the first injection. The other two were observed until recovery. None of the animals appeared ill after the single injection, although their temperatures rose 0.5°F. during the first 2 hours.

Assuming the circulating blood volume to have been a liter or less and the microorganisms evenly distributed at once, the injection of 1 cc. of culture would have produced a concentration of pneumococci in the blood of 1 million or more per cc. By actual count, blood drawn from the several dogs 2 minutes after injection yielded on culture from 170,000 to 800,000 colonies per cc. During the succeeding 28 minutes the colony counts fell rapidly, reaching a range of 3 to 80 at the end of that time (Table I). When the data for each dog are charted with time on the axis of abscissas and logarithms of the colony counts on the axis of ordinates, the resulting graphs are nearly linear (Fig. 1). It is apparent therefrom that the number of viable pneumococci disappearing from the circulation in unit time represented a nearly constant proportion of those free in the blood at the moment.

The graphs are not entirely straight but display a slight upward concavity, indicating a slight but progressive decline in the velocity at which bacteria were being removed from the blood stream. This decline in rate cannot be definitely correlated with a reduction in the pneumococcidal-promoting action of the serum or in the white blood count. That the clearing power of the circulation remained essentially intact 2 hours after the injection was shown by the equally rapid clearance of pneumococci after additional injections of culture. This result was observed in dog 1-57T which was given a second injection 2 hours after the first, and in dog 1-81T which received 4 injections at 2 hour intervals.

Clearing of Intravenously Injected Pneumococci from the Blood Stream of Pneumonic Dogs Not Spontaneously Bacteremic

It was pointed out in the first paper of this series (1) that natural antibodies against the pneumococcus usually persist undiminished in the blood of dogs

TABLE I

Clearing of Intravenously Injected Pneumococci from the Blood of Normal Dogs, in Vivo and in Vitro

(1)	(2)	(3)	(4)			(5)	(6)
Dog No.	Intervals at which blood samples taken after intravenous injection of pneumococci	No. of bacteria in circulating blood Colonies/cc.	No. of bacteria in blood withdrawn at 2, 5, and 15 min. intervals after injection, agitated in incubator for times shown in column (2), then cultured Colonies/cc. in			Pneumococcidal-promoting power of serum	White blood cell count
			2 min. sample	5 min. sample	15 min. sample	No. of pneumococci killed in serum-leucocyte mixture (0.5 cc.)	
1-39T	Zero*					10^5	25,000
	2 min.	200,000					
	5 “	30,000					
	15 “	480					
	30 “	3		0	0		
	1 hr.	0				10^4	9,400
	4 hrs.	0					13,700
	6 “	2	16	0	0		
	24 “	0	∞	0	0		
	3 days	0					
1-57T‡	Zero*					10^5	12,000
	2 min.	200,000					
	5 “	50,000					
	15 “	1,200					
	30 “	60	480	140	110		
	2 hrs.	0				10^5	6,600
	24 “	—	∞	0	0		5,200
1-81T‡	Zero*					10^5	6,700
	2 min.	170,000					
	5 “	9,000					
	15 “	400					
	30 “	80	320	68	12		
	2 hrs.	0					4,300
	24 “		∞	∞	0		
2-43T§	Zero*					10^5	7,500
	2 min.	800,000					
	5 “	110,000				10^4	7,200
	15 “	2,400					
	30 “	60	36,000	6,400	840	10^3	7,200
	2 hrs.	—	12,000	2,000	60		
	6 “		∞	36,000	1,300		
2-66T	Zero*					10^4	7,600
	2 min.	350,000					
	5 “	13,000				10^4	
	15 “	920					
	30 “	14	1,600	320	220		
	2 hrs.	5	2,800	40	40	10^4	6,500
	6 “	0	∞	300	1		
	24 “	0					17,000

∞, too many to count.

* Observations made immediately prior to the intravenous injection of culture.

‡ Injected again at 2 hours. Observation made during the few hours after the second injection are not shown in table but are described in Experiment 1.

§ Killed at end of 30 minutes.

having experimental pneumonia uncomplicated by bacteremia, and they do not disappear until the blood stream has been invaded. These findings were interpreted as indicating that bacteremia is not due primarily to a defect in the blood's antipneumococcal action but rather to a functional failure of an unknown barrier to the escape of pneumococci from the pulmonary lesion. If this conception be correct, one might expect that pneumococcemia artificially

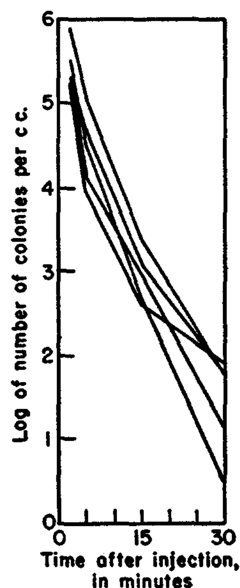

FIG. 1. Clearing of intravenously injected pneumococci from blood stream of normal dogs. Pneumococci from 1 cc. of culture injected intravenously at time zero. Numerical data given in Table I.

produced in pneumonic dogs would promptly subside. This hypothesis was tested in the following experiment.

Experiment 2 (*Table II*).—Three dogs (2-00T, 2-23T, 2-35T) were infected intrabronchially by the method of Terrell, Robertson, and Coggeshall (2) with 0.02 cc. of pneumococcus culture in 1 cc. of starch-broth medium. This dose results in a mortality of 7 to 8 per cent. 24 hours later when a lobar lesion was present, each animal was given an intravenous injection of pneumococci from 1 cc. of culture as in Experiment 1. The blood was sterile at that time.

A fourth dog (2-45T) was infected intrabronchially with 1 cc. of culture followed by 3 cc. of mucin, a dose producing a mortality of 50 per cent or more (3). When an intravenous injection was given 24 hours later, the pulmonary lesion occupied one-half the total lung field as shown by x-ray, the white blood count was 1,500, and the blood stream sterile.

Pneumococci, introduced intravenously into the three dogs having moderately severe pneumonia, left the circulation at the rate observed in normal dogs during the first half hour (Table II). However, in 2 cases bacteria per-

TABLE II

Clearing of Intravenously Injected Pneumococci from the Blood of Non-Bacteremic Dogs during Pneumonia, in Vivo and in Vitro

(1)	(2)	(3)	(4)			(5)	(6)
Dog No.	Intervals at which blood samples taken after intravenous injection of pneumococci	No. of bacteria in circulating blood	No. of bacteria in blood withdrawn at 2, 5, and 15 min. intervals after injection, agitated in incubator for times shown in column (2), then cultured			Pneumococcidal-promoting power of serum	White blood cell count
		Colonies/cc.	Colonies/cc. in			No. of pneumococci killed in serum-leucocyte mixture (0.5 cc.)	
			2 min. sample	5 min. sample	15 min. sample		
2-00T	Zero*	0				10^5	8,500
	2 min.	260,000					
	5 "	120,000					
	15 "	2,000					
	30 "	60	2,400	2,000	300		
	2 hrs.	15				10^5	6,900
	6 "	32	∞	∞	1,600		7,300
	24 "	50					11,000
	2 days	240					7,800
	3 "	2					
	4 "	0					
	5 "	0					
2-23T	Zero*	0				10^4	18,500
	2 min.	—					
	5 "	120,000					
	15 "	1,100					
	30 "	40	4,000	2,000	1		
	2 hrs.	5				10^5	7,800
	6 "	15	5,000	900	0		18,800
	24 "	10				10^5	19,000
	2 days	0					
2-35T	Zero*	0				10^5	10,400
	2 min.	300,000					
	5 "	50,000					
	15 "	600					
	30 "	90	40,000	1,600	36		
	2 hrs.	1	1,600	1,100	20	10^5	9,700
	6 "	14	∞	6,000	260		
	24 "						16,200
2-45T‡	Zero*	0				10^5	1,500
	2 min.	600,000					
	5 "	100,000					
	15 "	2,000					
	30 "	760	40,000	1,400	230		1,400
	2 hrs.	300	70,000	70,000	550		
	6 "	—	∞	∞	∞	10^4	1,000
	24 "	1,800					

∞, too many to count.

* Observations made immediately prior to the intravenous injection of culture.

‡ Died approximately 48 hours after the intravenous injection of pneumococci. The others recovered in 3 to 4 days.

sisted in small numbers in the blood for 1 to 3 days, an occurrence observed in none of the normal animals. A fourth dog (2-45T), which had a fulminating pneumonia accompanied by leucopenia, exhibited rapid clearing of the blood stream during the first 15 minutes. Thereafter, the rate of clearing in this animal was much slower, and at 24 hours bacteremia was increasing in degree. In none of the 4 pneumonic dogs was the pneumococcidal-promoting power of the serum significantly diminished 2 hours after the intravenous injection. Nor was there any decided reduction in white blood count except in one instance (2-23T) in which the count was high initially. In 2 cases the body temperature rose a little but the dogs did not appear more ill. In no instance was there a spread of the pulmonary lesion subsequent to intravenous injection which could be detected by serial x-ray examinations. Thus, artificial induction of bacteremia in the 3 animals having moderately severe pneumonia did not materially affect the course of the disease, and they recovered within the usual time of 3 to 4 days. The same was probably true in the case of the fourth animal since the large intrabronchial infecting dose employed is commonly followed by bacteremia and death.

Pneumococcidal Action of Blood in Vitro

(*a*) *Normal Blood.*—Before proceeding to study the behavior *in vitro* of blood of diseased dogs, it was considered necessary to obtain more detailed information concerning the rate with which pneumococci are killed in normal blood. For this purpose we used defibrinated blood according to the technique of Wu and Sia (4)[1] modified as outlined below.

Experiment 3 (*Fig. 2*).—One-tenth cc. quantities of the standard pneumococcus suspension in serial tenfold dilutions containing from 10 to 10^7 microorganisms were placed in a series of small tubes and to each was added 0.5 cc. of defibrinated blood at incubator temperature. Agitation of the mixtures in the incubator was begun 2 to 3 minutes later. At intervals similar to those used in the *in vivo* experiments, contents of the tubes were cultured in agar. Time was measured from the moment agitation started. A series of tubes for each dilution was prepared so that individual ones could be opened for culturing without interrupting the test.

With seedings of pneumococci varying in size up to 1 million per 0.5 cc. of blood, the colony count tended to fall geometrically and at a rate closely approximating that seen in the clearing of injected microorganisms from the circulation (Fig. 2). The same results was obtained with the blood of another dog.

The only known means by which normal blood destroys pneumococci is phagocytosis and intracellular digestion. That bacterial agglutination may have played a part in reducing colony counts in these tests is excluded by control observations on serum in the absence of eucocytes. The logarithmic progression evident in the declining counts is consistent with

[1] Somewhat earlier Ward (5) had demonstrated the pneumococcidal action of defibrinated blood of human beings.

what is known of the process of phagocytosis under the conditions of the experiment. Fenn (6), working with an agitated suspension of leucocytes and inanimate particles, found that collision between phagocyte and particle was attributable solely to chance, and that with a fixed number of leucocytes the frequency of collision, and thus the rate of phagocytosis, varied directly with the number of uningested particles.[2] Application of this conception to the case of encapsulated bacteria assumes that the rate of antigen-antibody reaction introduces no limiting factor. Such assumption may be justified by the evidence obtained by Mayer and Heidelberger (11) that union between pneumococcus polysaccharide and homologous antibody produced in the horse is at least 90 per cent complete within 3 seconds. This high reaction velocity does not necessarily apply in the case of natural immune bodies, of course. Another difficulty in concluding that chance of collision between leucocyte and bac-

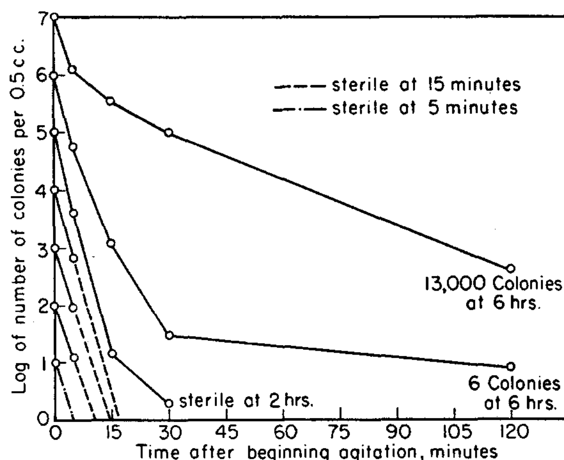

FIG. 2. Clearing of implanted pneumococci from normal dog blood *in vitro*. One-half cc. quantities of defibrinated blood inoculated with 0.1 cc. pneumococcus suspension in serial tenfold dilutions. The standard suspension was adjusted to contain approximately 10^9 microorganisms per cc. Control plating of 0.1 cc. of the seventh dilution yielded 10 colonies. The calculated numbers of bacteria implanted in the blood are shown at time zero. W.B.C. = 12,000.

terium determines the amount of phagocytosis arises out of the finding that rate of clearing is much the same in serum-leucocyte mixtures as in defibrinated whole blood (as seen in Experiment 4 below). Wide variation in the concentration of a third particulate constituent of the suspension, the erythrocyte, might be expected to influence the frequency with which the other two particles are free to collide with one another. Perhaps chemotaxis or some other factor, such as trapping[3] of the bacterium between leucocyte and erythrocyte, operates to nullify the difference in numbers of red cells interposed between phagocyte and bacterium.

When the size of the bacterial inoculum (10 million per 0.5 cc. of blood) exceeded by many times the complete killing power of the blood, clearing never-

[2] Chick (7) and others have shown that killing of bacteria in a disinfectant solution likewise obeys the law of mass action and resembles a monomolecular chemical reaction.

[3] Wood *et al.* (9, 10) have emphasized the importance of such effects in promoting phagocytosis.

theless proceeded rapidly at first although it slowed down decidedly after 5 minutes. A further observation of interest was that blood which could not destroy entirely an inoculum of 1 million microorganisms did kill, however, more than 99 per cent of a seeding 10 times larger. This behavior indicates that the larger the implantation, the more efficient was the pneumococcidal mechanism from the standpoint of total numbers killed. These quantitative features are seen in the results of the following experiment carried out with a serum-leucocyte mixture instead of defibrinated blood.

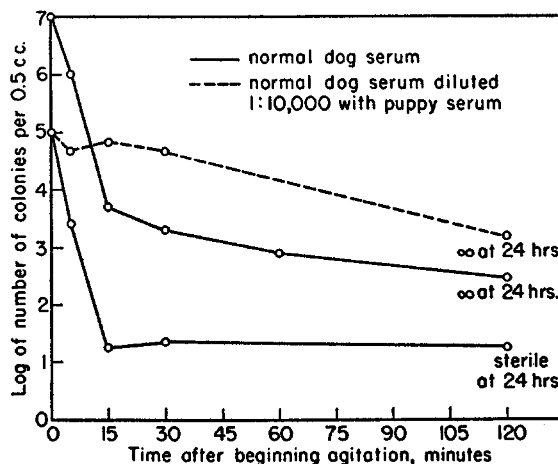

FIG. 3. Clearing of pneumococci from serum-leucocyte mixtures. Control culture of 0.1 cc. of the seventh tenfold dilution of the standard bacterial suspension yielded 10 colonies. The calculated numbers of bacteria implanted in the serum-leucocyte mixture are shown at time zero. W.B.C. = 11,000. Pneumococcidal-promoting power of the normal serum = 10^5; and of the diluted serum = 10^2.

(*b*) *Serum-Leucocyte Mixture.*—

Experiment 4 (*Fig. 3*).—The pneumococcidal action of a serum-leucocyte mixture was studied by the technique of Robertson and Sia (8), modified only in that tubes were cultured quantitatively at intervals during the course of the test.

Here again there was at the outset a rapid and striking reduction in number of microorganisms even when the size of the inoculum exceeded by at least 10 times the maximum killing power of the serum-leucocyte mixture (Fig. 3). A brief phase of rapid clearing was followed by a prolonged period of gradually declining colony counts. An implantation of 10 million bacteria was reduced to 5,000 in 15 minutes, but at the end of an hour 800 still remained, and at 2 hours, 290. Likewise, an inoculum of 100,000 was reduced to 18 in 15 minutes, but at the end of 2 hours the count was again 18. In only the second instance did the mixture finally become sterile. A repetition of the test with another serum-leucocyte mixture gave similar results.

The diminishing velocity of clearing which permitted long survival of a few microorganisms after millions had been quickly killed attracted our attention. Had chance of collision governed the rate of phagocytosis and had the pneumococci not multiplied, the observed deceleration would mean that as time went on fewer and fewer collisions were effective in promoting engulfment. In any case, the course of events reflected a progressive change in the bacterium or in the phagocytic system. Among possible alterations pertaining to the pneumococcus, the following were considered: (1) bacterial multiplication; (2) graded variation in virulence, a portion of the bacterial population having been highly resistant to destruction; (3) a limiting time element related to bacterial growth and consequent deleterious changes in the leucocytes. Experience with the growth of the pneumococcus in rabbit blood and dog serum would indicate that its rate of multiplication in these media is not sufficiently rapid, especially during the first hour after inoculation, to counterbalance the normal speed of clearance. The question of variation in virulence among members of the culture was examined by comparing with the standard suspension, the phagocytosis-resisting properties of a subculture of the surviving colonies from tubes which had failed to become sterile. No difference was observed. Experiment 4 was repeated with a 6 hour culture because of the possibly greater resistance of more actively growing bacteria. The results, however, were essentially the same as with the 16 hour culture. It remained then to determine whether changes within the medium adversely affecting the pneumococcidal activity of serum and leucocytes could account for the decline in the rate of diminution of pneumococci. To this end an equal amount of fresh serum-leucocyte mixture was added to the seeded mixture which had during the preceding 2 hours killed all but 290 of the 10 million pneumococci implanted. Neither acceleration of the clearing process nor final sterilization ensued. This observation was confirmed in further tests.

In regard to other factors relating to the opsonophagocytic system possibly responsible for the declerating course of clearing, the following were examined: (1) escape from leucocytes of ingested or partially ingested bacteria; (2) decrease in capacity of the cells to take up pneumococci; (3) a quantitative feature of the antibody-antigen reaction. The first possibility was explored because numerous Gram-positive diplococci apparently intact were readily identified within leucocytes after 2 hours' incubation of microorganisms in a serum-leucocyte mixture. No viable pneumococci could be recovered from such leucocytes by grinding them in a special mortar or by disrupting them in distilled water.

The ratio of numbers of bacteria to leucocytes with the heaviest inoculations employed did not exceed 2:1, and was usually 1:5 or lower. Because a leucocyte is known to take up many more than two opsonized diplococci, and because the addition of fresh white blood cells failed to accelerate the clearing process, it seems unlikely that the limit of phagocytic capacity of the cells was reached

in these tests. This conclusion conforms with the observation of Robertson and Sia (8) in their early work with serum-leucocyte mixtures that increasing the white cell concentration above 10,000 per c.mm. failed to produce any increased inhibition of pneumococcal growth.

The known characteristics of antigen-antibody reaction in specific precipitation provide an attractive explanation for some otherwise puzzling features of the antipneumococcal action of blood. Heidelberger and Kendall (12) and others have shown that antigen and antibody can unite in varying proportions depending on their relative concentrations in the medium; that a given amount of antibody combines with the largest amount of antigen toward the region of antigen excess, and that a proportionally larger amount of antibody is required to neutralize all of the antigen in the equivalence zone. Mudd, McCutcheon, and Lucké (13) in their review of the subject of phagocytosis call attention to the occurrence of zone phenomena in opsonization. Our observation of the blood's more efficient pneumococcidal action with the larger seedings would suggest that antibody combining with angtigen in the lower ratios is effective in opsonizing bacteria. The failure of additional serum to promote complete sterilization in a serum-leucocyte mixture which had previously destroyed all but a small fraction of a large implantation would be due, in similar terms, to considerably larger requirement for antibody in the equivalence zone in which no antigen exists uncombined. Analyzed further, the latter hypothesis implies that capsular antigen of pneumococci already engulfed by leucocytes continues to exert its normal influence in the antigen-antibody equilibrium of the medium as a whole. Further evidence is of course needed to clarify this phenomenon.

(*c*) *Bacteremic Blood.*—

Experiment 5 (*Fig. 4*).—Blood from dogs spontaneously bacteremic during the course of pneumonia was heparinized, distributed in a series of tubes, and treated in the same manner as the inoculated blood in Experiment 3 except that no pneumococci were added.

Graphs in Fig. 4 illustrate the behavior of incubated bacteremic blood. The several types of graph are representative of a large number of tests. In general, disappearance of the microorganisms proceeded more slowly than was observed in the case of normal blood seeded with comparable numbers of bacteria. Pneumococcidal-promoting power was low or absent in the filtered serum from these bacteremic bloods. The absence of demonstrable immune properties in such serum does not mean necessarily that none was present in the whole blood. Under these circumstances practically all of the available antibody would have been bound to the contained bacteria and none free in the medium.

When weak immune serum (normal dog serum highly diluted with puppy serum) was tested in a serum-leucocyte mixture, clearing was likewise slow and prolonged (Fig. 3). This behavior of a serum-leucocyte mixture low in antibody and of bacteremic blood depleted to a greater or less extent of im-

mune properies, would signify according to theories previously discussed that in the zone of extreme antigen excess opsonization was incomplete, and therefore relatively few of the collisions between bacterium and leucocyte led to phagocytosis.

On a few occasions bacteremic blood was capable of complete self-sterilization although its serum apparently possessed no pneumococcidal-promoting properties. It would seem that all of the antibody had been fixed to the bacteria and none was in excess in the serum. Four instances were encountered in which such blood destroyed also substantial additions of pneumococci (dog 2-06T, Fig. 4, represents an example).

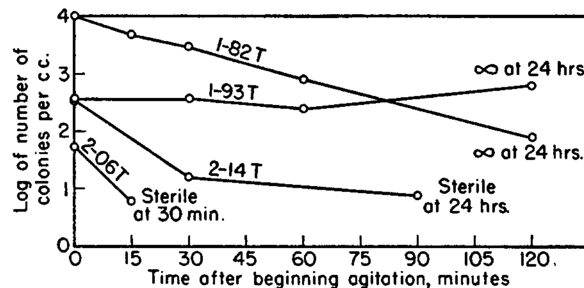

FIG. 4. Clearing of pneumococci from the shed blood of bacteremic dogs. Colony counts in freshly drawn blood are shown at time zero.

Dog 1-82T (Jan. 14, 1939), W.B.C. = 7,300; pneumococcidal-promoting power of serum = 0.

Dog 1-93T (Feb. 1, 1939), W.B.C. = 1,350; pneumococcidal-promoting power of serum = 10^1.

Dog 2-06T (Feb. 6, 1939), W.B.C. = 8,800; pneumococcidal-promoting power of serum = 0; additional pneumococci killed in heparinized blood = 10^3.

Dog 2-14T (Feb. 23, 1939); W.B.C. = 21,000; pneumococcidal-promoting power of serum = 10^5; additional pneumococci killed in heparinized blood = 10^4.

When the leucocyte count in bacteremic blood was severely depressed, although the serum may have retained some pneumococcidal-promoting power, the blood exhibited little tendency to clear (dog 1-93T, Fig. 4).

Blood obtained from dogs a few hours before death sometimes showed no inhibitory action against the contained microorganisms. In this respect it resembled normal rabbit blood which as a control medium in various phases of this work showed no antibacterial action whatever against the strain of pneumococcus employed, as would be anticipated from the earlier studies of Robertson and Sia (8) and Woo (14).

Direct Comparison of the Rate of Clearing in Vivo and in Vitro

To compare directly the rate of clearing *in vivo* and *in vitro* during the course of Experiments 1 and 2, blood was obtained from the intravenously injected

dog at intervals and parallel observations made on it after agitation in the incubator as described below. Coagulation was prevented by the use of heparin.

TABLE III

Clearing of Intravenously Injected Pneumococci from the Blood of Dogs Spontaneously Bacteremic during Pneumonia, in Vivo and in Vitro

(1)	(2)	(3)	(4)				(5)	(6)
Dog No.	Intervals at which blood samples taken after intravenous injection of pneumococci	No. of bacteria in circulating blood	No. of bacteria in blood withdrawn at 5, 15, and 30 min. intervals after injection, agitated in incubator for times shown in column (2), then cultured				Pneumococcidal-promoting power of serum	White blood cell count
		Colonies/cc.	Colonies/cc. in				No. of pneumococci killed in serum-leucocyte mixture (0.5 cc.)	
			Zero sample	5 min. sample	15 min. sample	30 min. sample		
2-75T	Zero*	22,000					<10	6,200
	2 min.	180,000						
	5 "	320,000						
	15 "	130,000						
	30 "	46,000	13,000	54,000	74,000			
	2 hrs.	25,000	3,400	75,000	9,000	9,800		
	6 "	14,000	80,000	250,000	18,000	30,000		
2-88T	Zero*	1,800					<10	4,100
	2 min.	700,000						
	5 "	390,000						
	15 "	16,000						
	30 "	2,900	240	100,000	3,200			
	2 hrs.	2,000	70	50,000	420	70		
	6 "	1,600	1,600	∞	10,000	1,600		
2-96T	Zero*	10,000					<10	6,900
	2 min.	900,000						
	5 "	650,000						
	15 "	100,000						
	30 "	40,000	6,000	16,000	8,000			
	2 hrs.	13,000	∞	∞	32,000	24,000		
	6 "	10,000			∞	∞		

∞, too many to count.

<10, no detectable pneumococcidal-promoting power.

* Observations made immediately prior to the intravenous injection of culture.

Experiment 6 (*Tables I and II*).—The 5 normal dogs employed in Experiment 1 and the 4 pneumonic dogs in Experiment 2 were studied in this manner. Each time that blood was drawn for culture during the first 15 minutes following intravenous injections of bacteria, an additional amount was taken for *in vitro* testing. The sample was distributed in a series of tubes which were then placed in the agitating device within 3 minutes. One tube from

each series was removed from the agitator and cultured at the same time the animal was bled for the half-hour, 2 hour, and 6 hour blood culture.

Blood withdrawn from dogs 2 minutes after the intravenous injection of pneumococci was capable of destroying from 93 to 99.8 per cent of the contained microorganisms during the succeeding 28 minutes, which closely approached the clearing rate *in vivo*. However, shed blood containing the very largest numbers of pneumococci failed to attain sterility. In this respect the pneumococcidal action of blood *in vitro* was definitely less effective than the clearing mechanism of the intact animal. Specimens taken at the 5th and 15th minutes, by which time the number of bacteria in the circulation had already diminished substantially, sometimes became sterile *in vitro*.

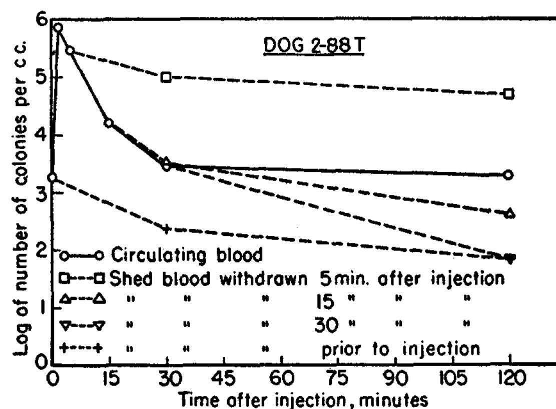

FIG. 5. Clearing of pneumococci from the blood of a dog bacteremic during pneumonia, *in vivo* and *in vitro*. Pneumococci from 1 cc. of culture injected intravenously at time zero. Number of bacteria present in the blood immediately prior to the injection is shown at time zero. Numerical data given in Table III.

Clearing of Intravenously Injected Pneumococci from the Blood of Dogs Spontaneously Bacteremic during Pneumonia

As has been shown, shed blood of dogs having bacteremia during the course of pneumonia is frequently capable of killing many and sometimes all the contained microorganisms, suggesting that the antipneumococcal function of the blood stream is preserved to a considerable degree under these conditions and is consequently capable of exerting some control on the extent of pneumococcemia. The circulation of such animals should possess, therefore, clearing action readily demonstrable by the technique used in non-bacteremic dogs. To learn whether this held true was the aim of the following experiment.

Experiment 7 (*Table III*).—Three dogs having bacteremia during the course of experimentally produced lobar pneumonia were injected intravenously with pneumococci from 1 cc. of culture as in Experiments 1 and 2 and studied in the same way. The intravenous injection was made 48 hours after intrabronchial infection when a multilobar pneumonic lesion

was well developed, the blood stream heavily invaded (more than 1000 colonies per cc.), the pneumococcidal-promoting power of the serum no longer measurable, and the shed blood unable to sterilize itself *in vitro*.

Introduction of additional pneumococci into the circulation produced only a transient increase in the height of bacteremia. The number of circulating bacteria fell rapidly during the first half hour, and within 2 to 6 hours the colony counts were approximately the same as prior to the intravenous injection (Table III). All the dogs died, as they invariably do with the grade of pneumococcemia existing at the beginning of the experiment (3).

Blood often cleared as fully *in vitro* as it did *in vivo* over the same length of time, and in some instances blood attained a lower bacterial concentration *in vitro* than in the circulation. The latter situation is interpreted as reflecting the continuous invasion of the blood stream from the lung. Fig. 5 shows graphically this relationship in one of the animals.

DISCUSSION

The foregoing observations have provided answers to certain of the questions which motivated this investigation and have shed some light on others. The finding that the pneumococcidal activity of the shed blood reflects to a remarkable degree the ability of the body to dispose of circulating pneumococci suggests that the blood plays the principal role in controlling the extent of bacteremia in the dog with experimental pneumonia. In support of this concept is the further evidence derived from this study (1) that the natural bactericidal power of the blood tends to persist throughout the course of the disease and shows appreciable diminution only when pneumococcemia becomes extreme and when the number of circulating leucocytes falls below a critical level, and (2) that the blood possesses an immense reserve capacity for disposing of pneumococci as revealed by the rapid disappearance of microorganisms from both the circulating and shed blood following repeated intravenous injections of large quantities of pneumococcus culture.

The role played by natural humoral immune substances in the prevention and control of bacteremia is only partially elucidated by the results of our experiments. Observations made on dogs during the course of experimental pneumonia, recorded in Paper I, showed that while no bacteremia prevention effect could be ascribed to the presence of antipneumococcal immune bodies, their demonstrable occurrence in the blood was found to be definitely related to limitation of numbers of circulating pneumococci. Similar evidence resulted from the intravenous injection of pneumococci into dogs with experimental pneumonia as exhibited in Tables II and III and Fig. 3 of the present paper. While diminution and loss of humoral immunity were usually associated with increasing bacteremia, in certain cases blood invasion remained minimal despite disappearance of demonstrable serum immune properties. Tests on the

shed blood of such animals showed that it possessed considerable pneumococcidal power. The explanation offered for this phenomenon, namely that all the available antibody had been absorbed on the circulating pneumococci, would not account, however, for several instances in which the blood lacking humoral immune activity was found capable not only of sterilizing itself but also of killing substantial additions of pneumococci. That there exists a gap in our knowledge of the bactericidal mechanism is further evidenced by tests on the shed blood of heavily bacteremic dogs injected with large quantities of pneumococcus culture. Such blood, lacking demonstrable humoral immunity, nevertheless was capable of killing great numbers of microorganisms, although the effect lasted only a few hours (Table III).

The rapid disappearance from the circulating blood of large quantities of intravenously injected pneumococci suggests that the number of microorganisms constantly escaping from the pulmonary lesions in the bacteremic state must be greatly in excess of that revealed by blood culture. However, our observations provide no information as to the rate of such escape nor do they yield an inkling as to why and how bacteremia occurs. An approach to this problem will require first, exploration of the path of escape of pneumococci from the lung lesions, then elucidation of the nature of the lung-blood barrier or barriers.

That the body possesses more than one mechanism for disposing of bacteria which have gained entrance into the blood stream seems clear from the work of numerous investigators. Studies on the fate of pathogenic bacteria injected into the blood of various animal species have shown that with few exceptions such microorganisms disappear rapidly from the circulation. This is true for both virulent and avirulent bacteria and for naturally resistant and susceptible animals. Observations on pneumococcemia in the rabbit (15, 16, 18, 19), an animal which exhibits very little natural resistance to infection with virulent strains of pneumococci and lacks blood bactericidal power, have demonstrated that these microorganisms injected intravenously, in quantities greatly in in excess of the lethal dose, were removed quickly and almost completely from the circulating blood. Such restraint of bacteremia was only temporary, however, since the rabbits thus treated always died of an overwhelming pneumococcemia. On the other hand, injection of similar strains of pneumococci into pneumococcus-resistant animals, *e.g.* the dog, was followed by an even more rapid disappearance and permanent elimination of the microorganisms as shown by our results and those of others (17). Rabbits immunized actively against pneumococci reacted in essentially the same manner as the dog and exhibited pneumococcidal activity in the blood (15).

Investigation of the sites of removal of intravenously injected microorganisms, carried out principally in the rabbit, have shown that they were mobilized chiefly in the liver, spleen, and lungs. The relative importance of these several

organs as collecting loci appeared to vary with the kind of bacteria and the species of animal employed (15–17). The fate of such mobilized bacteria was found to depend on numerous factors: dosage, virulence, relative immunity, etc. Under conditions favorable to the animal the microorganisms have been observed to be engulfed and destroyed by the fixed phagocytes and mobilized leucocytes. This process is accelerated by the presence of natural immune substances but phagocytosis by sessile and wandering macrocytes, as well as by polymorphonuclear leucocytes, may occur without the aid of opsonins as shown by Wood and collaborators (9, 20) in their studies on surface phagocytosis. Thus while the shed blood of a pneumococcus-susceptible animal, *e.g.* the rabbit, lacking opsonins, fails to exhibit bactericidal activity, killing of these microorganisms could still occur in the reticulo-endothelium–rich organs and in the capillaries.

It would seem then that the mechanism by which the susceptible animal clears its blood stream of injected pathogens differs in certain respects from that of the resistant one, at least for the pneumococcus. In the rabbit, for example, the phagocytic cells of the reticulo-endothelial system, especially those of the liver and spleen, appear to take the dominant role, with bactericidal activity of the blood playing a minor part. In a highly resistant animal such as the dog, on the other hand, the pneumococcus-killing power of the blood probably constitutes the first line of defense and the reticulo-endothelial phagocytes act as a supplementary mechanism.[4] The latter may, however, become the principal clearing agent if the pneumococcidal action of the blood fails. The fact that disappearance of injected pneumococci from the circulating blood was, in most of our animals, somewhat greater than that observed to occur in the shed blood, suggests participation of the fixed tissue phagocytes in this function.

Previous observations by the authors and others on changes occurring in the antipneumococcal activities of the blood during the course of human lobar pneumonia, while much less extensive, are essentially similar to those of the present studies on the dog, suggesting that the mechanism controlling the process of bacteremia is the same in the two species.

SUMMARY

With the purpose of ascertaining the influence exerted by the pneumococcidal activity of the blood on the course of bacteremia occurring in experimental canine pneumococcal pneumonia, a study was made of the rates at which intravenously injected pneumococci disappeared from the circulation and the shed blood of diseased dogs. Preliminary studies on normal animals showed

[4] Recent observations by Martin, Kirby, and Holland (21) would contradict this conclusion, as regards the staphylococcus. They found that staphylococci, injected continuously into the blood stream of dogs, were removed from the circulating blood principally in the splanchnic organs and little or not at all in the peripheral circulation.

that blood containing hundreds of thousands of pneumococci per cc. immediately after injection usually became sterile or nearly so within an hour's time. Simultaneous observations carried out on the blood *in vitro* showed an analogous rapid disappearance of the microorganisms, although the effect was not quite as marked. Similar tests on non-bacteremic dogs with pneumonia revealed essentially the same ability of the body to dispose of large numbers of circulating pneumococci. The shed blood likewise exhibited marked bactericidal power.

The occurrence of bacteremia during pneumonia did not retard greatly the rate at which injected pneumococci disappeared from the circulation, as compared with the non-bacteremic state. After several hours the numbers of circulating microorganisms were approximately the same as prior to the intravenous injection. Blood *in vitro* often cleared as fully as it did *in vivo* over the same length of time.

Studies on the role played by humoral immune substances in the bactericidal action of the blood showed that while their presence was necessary for maximum killing power, and that bacteremic blood lacking humoral immune properties was rarely capable of self-sterilization *in vitro*, nevertheless such blood often retained considerable bactericidal potency as shown by its ability to reduce materially the numbers of pneumococci added to it. This phenomenon is discussed.

The marked pneumococcidal capacity of the blood exhibited by dogs with experimental pneumococcal pneumonia and its persistence during bacteremia suggest that this constitutes the principal mechanism for limiting the degree of blood invasion. The similarity of the findings in canine and human pneumococcal lobar pneumonia is pointed out.

BIBLIOGRAPHY

1. Robertson, O. H., Hamburger, M., Jr., and Gregg, L. A., *J. Exp. Med.*, 1935, **97**, 283.
2. Terrell, E. E., Robertson, O. H., and Coggeshall, L. T., *J. Clin. Inv.*, 1933, **12**, 393.
3. Robertson, O. H., and Fox, J. P., *J. Exp. Med.*, 1924, **69**, 229.
4. Wu, C. J., and Sia, R. H. P., *Proc. Soc. Exp. Biol. and Med.*, 1932, **29**, 786.
5. Ward, H. K., *J. Exp. Med.*, 1930, **51**, 675.
6. Fenn, W. O., *J. Gen. Physiol.*, 1921, **3**, 439, 465, 575.
7. Chick, H., *J. Hyg.*, Cambridge, Eng., 1908, **8**, 92.
8. Robertson, O. H., and Sia, R. H. P., *J. Exp. Med.*, 1924, **39**, 219.
9. Wood, W. B., Jr., Smith, M. R., and Watson, B., *J. Exp. Med.*, 1946, **84**, 387.
10. Wood, W. B., Jr., and Smith, M. R., *Science*, 1947, **106**, 86.
11. Mayer, M., and Heidelberger, M., *J. Biol. Chem.*, 1942, **143**, 567.
12. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1929, **50**, 809; 1935, **61**, 563; 1935, **62**, 697.
13. Mudd, S., McCutcheon, M., and Lucké, B., *Physiol. Rev.*, 1934, **14**, 210.

14. Woo, S. T., *J. Exp. Med.*, 1926, **43,** 623.
15. Wright, H. D., *J. Path. and Bact.*, 1927, **30,** 185.
16. Cannon, P. R., Sullivan, F. L., and Neckermann, E. F., *J. Exp. Med.*, 1932, **55,** 121.
17. Bull, C. G., *J. Exp. Med.*, 1916, **24,** 7.
18. Teale, F. H., *J. Immunol.*, 1935, **38,** 133.
19. Martin, S. P., and Kirby, G. P., *J. Exp. Med.*, 1950, **92,** 45.
20. Wood, W. B., Smith, W. R., Perry, W. D., and Berry, J. W., *J. Exp. Med.*, 1951, **94,** 521.
21. Martin, S. P., Kirby, G. P., and Holland, B. C., *Proc. Soc. Exp. Biol. and Med.*, 1949, **72,** 63.