CHANGES IN HUMORAL IMMUNITY OCCURRING DURING THE EARLY STAGES OF EXPERIMENTAL PNEUMOCOCCUS INFECTION*

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While much has been learned concerning the appearance of immune substances in the blood at the time of recovery from lobar pneumonia and experimental pneumococcus infection, relatively little is known about changes in humoral immunity occurring during the early phases of the disease. That such changes occur and have an important bearing on the inception and evolution of the infection seems highly probable in the light of recent experimental evidence as to the nature of natural resistance to pneumococcus infection (1).

The findings of earlier workers in this field are inconclusive . This is to be explained presumably by the use of methods lacking sufficient delicacy for the detection of normal anti-pneumococcus substances in the blood stream. Wolf (2), Rosenow (3), Tunnicliff (4), and others, testing phagocytic activity by the determination of the opsonic index (Wright), agreed for the most part that the opsonic index was low in severe pneumonia cases at the height of the disease and was followed by a slight rise at the time of crisis. In fatal cases there was a fall in the opsonic index before death. However, these workers used avirulent strains of pneumococci in their tests; whereas the organisms isolated from pneumonia patients were almost invariably virulent and found to be resistant to the opsonic action of the serum. Other workers (5), (6), (7), studying the humoral changes in lobar pneumonia, were unable to demonstrate opsonins, agglutinins, or mouse protective substances, in the blood stream until about the time of crisis. Bull (8), on the other hand, studying the natural resistance of the dog to pneumococcus infection, was able to demonstrate clearing power of normal dogs blood for highly virulent pneumococci. He injected dogs with virulent pneumococcus cultures intravenously and by plating samples of blood at short intervals observed a rapid decrease in the number of pneumococci in the blood stream following injection.

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In fatal cases this diminution was only temporary and was followed by an increase and death from septicemia. He inferred that the clearing of the blood was due to agglutination and the phagocytic action of the circulating leucocytes and the cells of the reticulo-endothelial system, although neither opsonins nor agglutinins could be demonstrated in the dog's serum until the sixth or seventh day of the disease.

Tillett (9), working with Type III pneumococcus, using a similar technique, injected rabbits intravenously and showed that avirulent or R forms were rapidly taken out of the blood stream. With strains of slight virulence, there was a decrease and a subsequent rise in the number of circulating pneumococci. When highly virulent strains were used, there was no diminution but a steady increase in the bacteremia until death of the animal. With the usual methods employed for the demonstration of opsonins and agglutinins, Tillett failed to find evidence of humoral immune properties sufficient to account for the rabbit's ability to destroy the avirulent pneumococci.

Recently Robertson and Sia (10) (11), studying the natural resistance of animals to pneumococcus infection, found by an especially devised technique that the serum and leucocytes of pneumococcus resistant animals such as the dog, cat, pig, etc., possessed marked pneumococcidal properties for highly virulent strains of pneumococci while the blood of pneumococcus susceptible animals as the rabbit and guinea pig was lacking in this action. This property of the serum and leucocytes of resistant animals was found to depend on the presence of opsonins and agglutinins which could be demonstrated by employing a relatively large ratio of serum to microorganisms (1).

The technique of Robertson and Sia, by which it is possible to demonstrate constantly natural humoral immune bodies in the blood of certain animal species, provides a means of studying changes in the circulating defense mechanism during the inception and evolution of pneumococcus infection. The object of the present study has been primarily to follow the early changes in the humoral immune substances of the dog and cat during experimental pneumococcus infection.

Methods

Infection: Normal dogs and cats were infected by intrapleural or intraperitoneal injections in the first experiments and by intrabronchial insufflation in the later ones. A virulent Type I strain and a virulent Type II strain of pneumococcus originally isolated from pneumonia cases were used. The virulence was maintained by frequent passage through rabbits.

Serum: A blood specimen was drawn before the animal was infected and daily specimens were taken until death or recovery. The blood was allowed to cool in

the ice box and the serum was withdrawn later. Blood cultures and plate counts were done on most of the animals. All sera that contained pneumococci were filtered through an especially designed filter.[1] The sera were preserved in the ice box in bottles containing carbon dioxide.[2] All sera from one animal were tested at one time not later than seven days after the first blood specimen was drawn.

Opsonic Test: The method described by Robertson and Sia (1) was followed closely. Homologous leucocytes were obtained from aleuronat exudates withdrawn from the pleural cavity 15 to 18 hours after injection of the aleuronat. Type I pneumococcus was used in the lag phase of growth and Type II pneumococcus in the active growth phase. The pneumococci were sensitized for periods of ½ hour for actively growing cultures and one hour for cultures in the lag phase in the serum to be tested. A ratio of 50 parts of serum to one part of pneumococcus suspension was employed. The organisms were then sedimented by centrifugation at high speed for ¾ hour; the serum was removed and the pneumococci were taken up in sufficient Locke's solution to make a suspension somewhat more concentrated than that originally added. Wright's capillary pipette mixtures were made using equal parts of sensitized pneumococcus suspension, 1 to 5 normal serum, and a standard leucocyte suspension. The pipettes were incubated 45 minutes and smears were made which were stained with Cross' stain. 100 leucocytes were counted and the percent showing phagocytosis noted.

Pneumococcidal Test: The pneumococcidal promoting substances of the serum were tested by the method described by Robertson and Sia (10). 0.1 cc. dilutions of an actively growing pneumococcus suspension were added to small tubes containing 0.3 cc. serum and 0.1 cc. of a standard leucocyte suspension. The tubes were sealed with corks dipped in paraffin and rotated on an agitator for 15 to 18 hours. Readings were made by determining the amount of methemoglobin formed at the end of 18, 42, and 72 hours. Smears were then made to determine the survival of pneumococci.

EXPERIMENTAL

Generalized Pneumococcus Infection

Experiment I: Cat No. 1, weighing 4000 grams, was bled 10 cc. from the heart and four hours later 0.5 cc. Type II pneumococcus culture was injected intrapleurally. 18 hours subsequently the cat's temperature was 41°C. and it appeared ill. Death occurred on the fourth day. Autopsy revealed peritonitis and a massive empyema of both pleural cavities. The lungs were not consolidated. Culture of the heart's blood was positive for penumococci. The collected sera were filtered to eliminate any pneumococci present and the opsonic activity of the serum was determined.

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[1] Small pressure filters were made, using the Seitz principle.

[2] According to McAlpine and Valley (12) alexin or complement can be preserved for a long period of time in an atmosphere of CO_2.

The results are shown in Figure I. The serum of the cat drawn before infection showed marked opsonic activity; 67 per cent of the leucocytes counted showed phagocytosis. 18 hours after infection

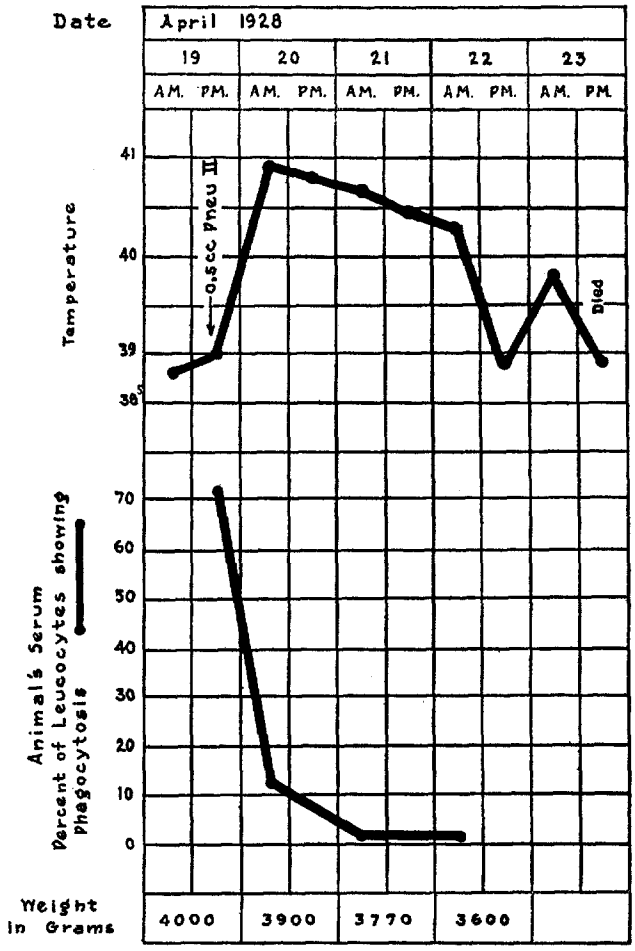

FIGURE I. Cat No. 1. Experimental pneumococcus infection following the intrapleural injection of 0.5 cc. Type II pneumococcus culture.

the opsonic titer had diminished markedly; only 12 per cent of the cells showed phagocytosis. After 48 hours this serum property had disappeared.

Experiment II: Dog No. 1, weighing 20.9 kg., was bled 15 cc. from the heart and 4 cc. of an actively growing Type I pneumococcus culture were injected into the right pleural cavity. The next morning the dog appeared sick and the temperature had increased to 41°C. Blood cultures at 18 hours and 42 hours after

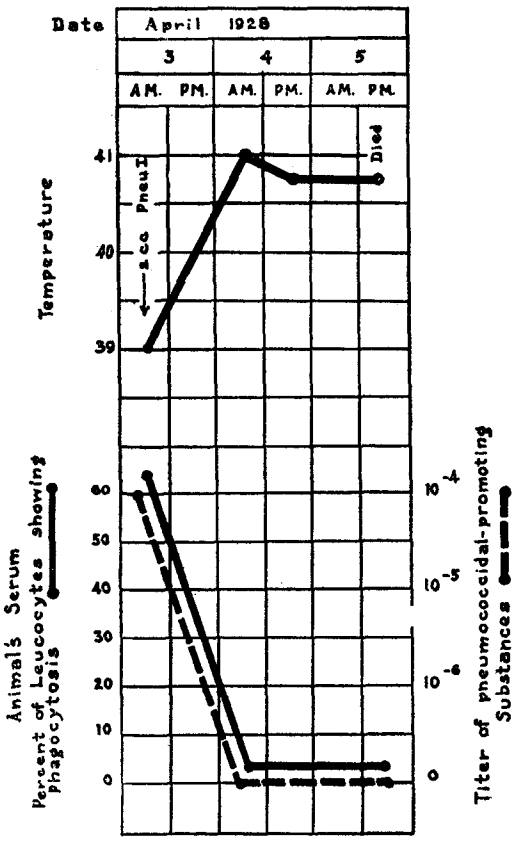

FIGURE II. Dog No. 1. Experimental pneumococcus infection following the intrapleural injection of 2 cc. Type I pneumococcus culture.

infection were positive for pneumococci. The dog died on the third day. Autopsy revealed a massive empyema of both pleural cavities. The lungs were not consolidated.

Before infection the serum contained a high concentration of opsonins. Phagocytosis was noted in 65 per cent of the leucocytes

counted with 60 per cent showing five pairs or more of pneumococci. 18 hours after the inception of infection the opsonic content of the serum had dropped to a point where only 5 per cent of the leucocytes showed phagocytosis with no cells containing more than one or two pairs of pneumococci (Figure II). The pneumococcidal promoting action of the serum showed a similar fall in activity (Table I). The serum (0.3 cc.) taken before infection was capable of causing destruc-

TABLE I

Effect of Pneumococcus Infection on the Pneumococcidal-Promoting Activity of the Serum

Dog serum 0.3 cc. + normal dog leucocytes 0.1 cc. + pneumococcus suspension 0.1 cc.

Kind of serum	Amount of standard suspension		Growth as shown by color changes			Survival of pneumococci stained film
			17 hrs.	42 hrs.	72 hrs.	
		cc.				
Serum before infection	10^{-2}	(0.01)	+++	++++	++++	+
	10^{-3}	(0.001)	++	+++	++++	+
	10^{-4}	(0.0001)	0	0	0	0
	10^{-5}	(0.00001)	0	0	0	0
	10^{-6}	(0.000001)	0	0	0	0
Serum 18 hrs. after	10^{-4}	(0.0001)	++++			+
	10^{-5}	(0.00001)	++++			+
	10^{-6}	(0.000001)	++++			+
	10^{-7}	(0.0000001)	++++			+
Serum 42 hrs. after	10^{-5}	(0.00001)	++++			+
	10^{-6}	(0.000001)	++++			+
	10^{-7}	(0.0000001)	++++			+

tion in the serum-leucocyte mixture of approximately 100,000 pneumococci (10–4 of the standard suspension) but 18 hours after infection it had lost this property.

Experiment III: Dog No. 2, weighing 8.2 kg., was bled 15 cc. from the jugular vein and 2 cc. of an actively growing culture of Type I pneumococcus were injected into the trachea by a needle and syringe. The dog was held so that the culture would run into the right lung. 18 hours later the dog's temperature had risen to 41.1°C. and it appeared very toxic. Death occurred on the third day. Autopsy

revealed an empyema of both pleural cavities. The lobes of the right lung were well collapsed and markedly congested. The left lung resembled the right except that it was more air containing. Microscopical sections showed congestion of the alveolar walls but no leucocytic exudate was present.

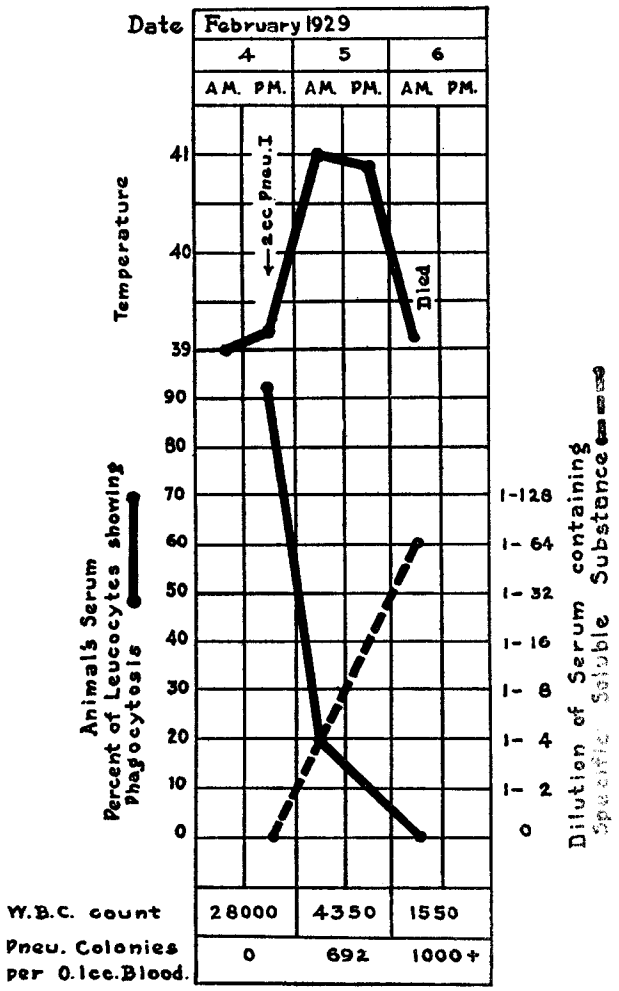

FIGURE III. Dog No. 2. Experimental pneumococcus infection following the intratracheal injection of 2 cc. Type I pneumococcus culture.

The results are shown in Figure III. At the end of 18 hours the opsonins were reduced but there were still enough present to cause a

slight degree of phagocytosis although approximately 6000 pneumococci per cc. were circulating in the blood stream and a demonstrable quantity of specific soluble substance was present in the serum. At the end of 42 hours the opsonins had disappeared completely while the number of pneumococci and amount of soluble substance in the serum had increased. The leucocyte count was reduced from 28,000 before infection to 1,550 on the day of death.

These three experiments, with others that followed a similar course, show that with an overwhelming pneumococcus infection accompanied by early blood invasion there is a rapid decrease in the concentration of humoral immune bodies which disappear entirely by the time of death. In other experiments in which animals recovered after a moderately severe generalized infection this same early diminution of humoral immune substances was observed to occur but the concentration of immune bodies began to rise with the onset of recovery which usually occurred about the fourth day. These immune bodies, appearing in the blood at the time of recovery, are of the so called acquired type as shown by Robertson and his co-workers (13).

In order to determine the nature of this decrease in humoral immune substances in a severe infection, a dog was injected with the filtrate of a 24 hour culture of pneumococcus. According to Avery and Heidelberger (14) the filtrate at this stage contains very little of the nucleoproteins of the pneumococcus but a considerable amount of the specific soluble substance which they have shown to be a carbohydrate, possibly a polysaccharide derived from the capsule of the cell. The experiment follows:

Experiment IV: Dog No. 3, weighing 16 kg., was bled 15 cc. from the heart and 50 cc. of filtrate from a 24 hour Type I pneumococcus culture were injected intravenously at 2 P.M. At 3 P.M. a sample of blood was drawn. At 4 P.M. 100 cc. of filtrate were injected intraperitoneally. Blood samples were drawn at 18 and 40 hours after injection.

The results are shown in Table II. There was a decrease in the opsonic action in the sample drawn one hour after injection. After 18 hours there was a slight return toward the initial opsonic titer but it did not return to normal until 40 hours after the filtrate injection. This diminution in opsonic activity is presumably due to the combina-

tion of the specific soluble substance with the natural immune bodies. Sia (15) using normal serum-leucocyte mixtures found that the presence of a very small amount of the purified soluble substance of the homologous type markedly altered the conditions in the mixture so that even a small number of avirulent pneumococci were enabled to grow in the serum and leucocytes of animals which possess the power to destroy ordinarily such pneumococci in relatively large numbers.

The opsonins in these experiments were highly type specific. The blood of animals infected with Type I pneumococcus did not show a decrease in opsonins when tested with a Type II organism and vice versa. The filtrate injections caused only a decrease in the opsonins for that specific type.

TABLE II

Effect of Intravenous Injection of Culture Filtrate on Opsonic Properties of the Serum

Kind of sensitizing serum	Degree of phagocytosis	
	Per cent of leucocytes showing phag.	Per cent showing 5 pairs or more
Serum before injection	78	66
One hour after injection	37	7
18 hours after injection	59	29
48 hours after injection	75	52

There has been evidence which has led some investigators (8) to think that a septicemia occurs, not because the natural immune substances have become exhausted, but because the organisms have become adapted to their host and have increased in virulence. In several dogs pneumococci were isolated from the blood stream at different stages of the septicemia and their susceptibility to phagocytosis compared with that of the organism originally used. No differences between them were found.

Localized Pneumococcus Infection

(*Lobar Pneumonia*)

A generalized and overwhelming infection as in the experiments above described is not the type of infection found characteristically

in lobar pneumonia. Although there may be a transient bacteremia and in some fatal cases a persistent bacteremia, lobar pneumonia, typically, is a localized pneumonic process. Lamar and Meltzer (16) produced with varying success, a lobar consolidation in dogs by injecting the pneumococcus culture deep into a bronchus by using a tracheal catheter. As a rule the dogs either developed a very light infection or the infection terminated fatally with empyema and a septicemia. With this in mind we attempted to produce a severe infection, yet an infection localized in the lungs. A similar method was employed except that the pneumococci were first suspended in a viscous medium (16 per cent gelatin broth). Because of the high degree of virulence for dogs, from 1 to 3 cc. of culture were sufficient to produce an infection. Five dogs out of nine treated in this manner developed a localized pneumonic infection. The other four died from septicemia and empyema.

Experiment V: Dog No. 4, weighing 17.6 kg., was bled 15 cc. from the jugular vein then etherized and a number 8 tracheal catheter was inserted as far as possible into a bronchus. Type I pneumococci from 3 cc. of an 18 hour culture were suspended in 16% gelatin broth solution and injected into a bronchus through the catheter. The next morning the dog appeared ill. The temperature had risen from 38.5°C. to 39.9°C. There was a marked cough, forced expiration, and an increased respiratory rate. X-ray of the chest taken on the third day showed consolidation of the three right lobes and the upper left lobe. The condition of the dog became progressively worse until death occurred on the sixth day. At autopsy the pleural cavities contained no fluid. The right lung was consolidated except for the lower half of the lower lobe which was air-containing. The left upper lobe was congested but air-containing. The left lower lobe appeared normal. The consolidated lobes were comparatively firm and gray in appearance. There was no fibrinous exudate covering the surface. On the cut surface was a grayish thick exudate. Microscopical sections revealed many polymorphonuclear leucocytes within the alveoli. There was only a slight amount of fibrin present. The heart's blood was negative for gram positive diplococci.

The results of this experiment are shown in Figure IV. The most important observation made was the fact the dog died with a negative blood culture. There was an early blood invasion noted 18 hours after infection but the remaining blood cultures proved negative. The pneumococcidal promoting power of the blood did not diminish to

an appreciable extent throughout the course of the infection. The opsonic properties of the serum corresponded in general with the pneumococcidal titers. Evidently the humoral immune substances in

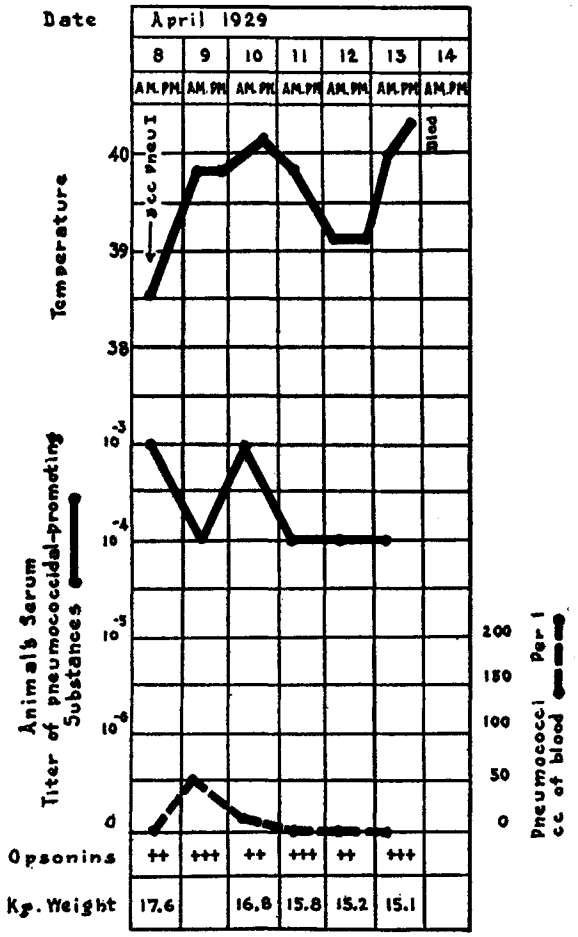

FIGURE IV. Dog No. 4. Experimental pneumococcus infection following the intrabronchial injection of 3 cc. Type I pneumococcus culture.

this case were sufficient to prevent invasion of the blood stream but, on the other hand, did not prevent the spread of the infection within the lung which was lobar in distribution.

Experiment VI: Dog No. 5, weighing 16 kg., was bled 15 cc. from the jugular vein, etherized, and pneumococci Type I from 2 cc. of culture were suspended in 16% gelatin broth solution and injected deep into a bronchus by intrabronchial insufflation. The next morning the dog appeared quiet, would not eat, and its

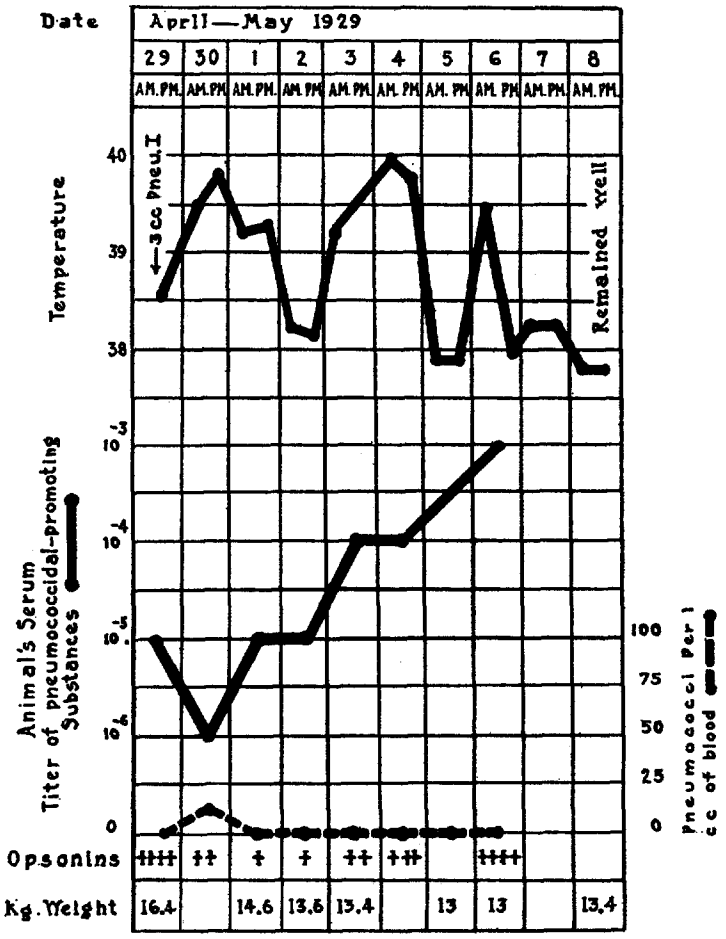

FIGURE V. Dog No. 5. Experimental pneumococcus infection following the intrabronchial injection of 3 cc. Type I pneumococcus culture.

temperature was slightly elevated. X-ray showed marked consolidation of the right lower and middle lobes. The third day the respiration had increased to 92 per minute, there was marked dullness on the right side to percussion, and on auscultation marked bronchial breathing was heard on the same side. On the seventh day the temperature subsided and the condition of the dog improved.

As in the previous case, there was a slight early blood invasion which did not persist after the second day (Figure V). The pneumococcidal promoting action of the serum was slightly decreased after 18 hours but there was still an appreciable degree present. On the third day the titer of this serum property had returned to normal and showed a further increase as long as the blood specimens were taken. Tests for opsonic activity showed a more marked decrease in this property but it followed a similar course.

Experiment VII: Dog No. 6, weighing 22 kg., was bled 15 cc. from the jugular vein, anesthetized with ether, and 3 cc. of Type I pneumococcus suspension injected deep into a bronchus. The next morning the dog appeared quite sick. The temperature was elevated. X-ray revealed a consolidation of the right lower lobe. The condition remained about the same until the fourth day when signs of fluid developed in the right chest. The dog died on the seventh day. Autopsy revealed a thick grayish fluid in the pleural cavity of each side. The pericardial cavity contained about 75 cc. of like material. Stained smear showed innumerable gram positive diplococci. The lobes of the right lung were well collapsed and dark red in color. The left lower lobe was congested but air-containing. The left upper lobe appeared normal. No true consolidation was seen. Microscopical sections from the lobes of the right lung showed congestion of the alveolar walls and red cells in some of the alveolar spaces. Very few leucocytes were seen.

The humoral immune changes occurring in this dog differed considerably from those in the preceding animals. 18 hours after infection the immune substances had not decreased (Figure VI) although there were 86 pneumococci per cc. in the blood. After 42 hours however, there was a marked reduction in the pneumococcidal-promoting substances and a slight diminution in the number of organisms per cc. of blood. By the fourth day of the disease the concentration of immune bodies had shown a rise while the number of circulating pneumococci had fallen to only 16 per cc. of blood. The following day when there were definite signs of fluid in the chest, the titer of the pneumococcidal-promoting substances was back to normal but there was a marked increase in the bacteremia; 280 organisms per cc. of blood were noted. The bacteremia increased until death although the pneumococcidal-promoting substances were still present.

In attempting to correlate the findings in this animal with the preceding observations, account must be taken of the type of lesion pres-

ent in the lung. Judging X-rays taken 24 hours after the onset of the infection there was a definite beginning localization of the pneu-

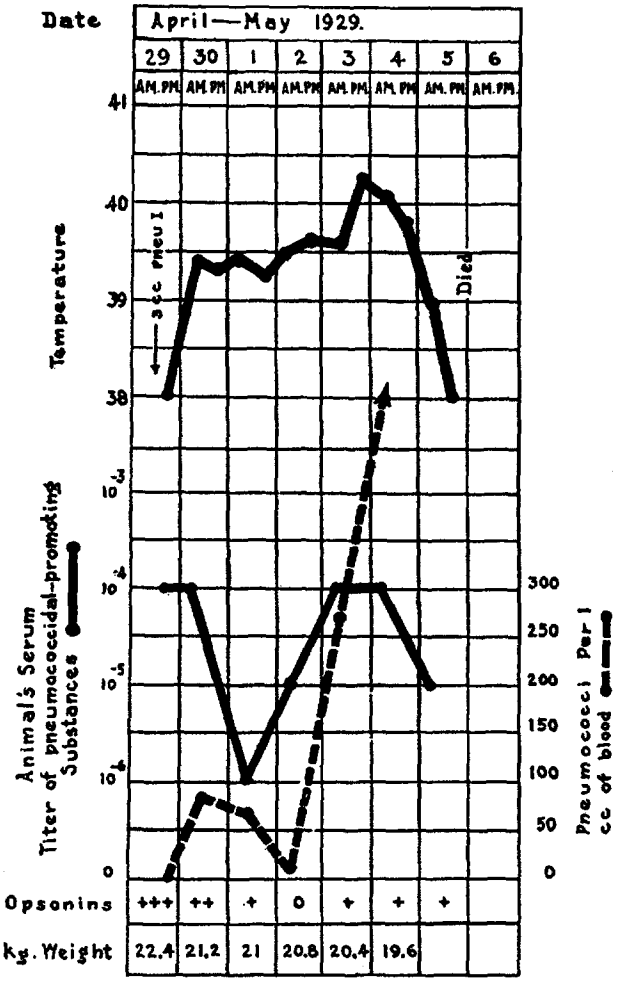

FIGURE VI. Dog No. 6. Experimental pneumococcus infection following intrabronchial injection of 3 cc. Type I pneumococcus culture.

monic process. While blood invasion at this stage was somewhat more marked than in dogs (3) (4), it was by no means as pronounced as in those animals suffering from a generalized pneumococcus infection.

As in the other dogs with lobar pneumonia this initial blood invasion diminished but not as quickly nor did the pneumococci disappear from the blood completely. From this it could be inferred that localization of the lung process did not develop as in the dogs with true lobar pneumonia. The lack of definite pneumonic consolidation found at autopsy bore out this assumption. The terminal state of the blood in which large numbers of pneumococci were circulating in the presence of a considerable concentration of pneumococcidal-promoting substances is not susceptible to a satisfactory explanation on the basis of the data available. It would seem most probable, however, that the phagocytic cells of the body were failing to function adequately. While leucocyte counts were not made in this animal, observations on other dogs with overwhelming pneumococcus infection showed a great reduction in the number of circulating leucocytes. The onset of empyema may have produced a depression of functional activity of the phagocytic cells of the body as well. The excess of antibody over antigen might be accounted for by the fact that at this stage of the disease, 4th to 5th day, the immune substances present were of the acquired type and being produced in a concentration much greater than that of the normal antibodies and yet one would expect that if the immune substances were active the pneumococci would be agglutinated and swept out of the blood stream as shown by Bull (8). An alternative possibility could be the presence of some unknown factor operating to prevent the union of antigen with antibody. That the invading pneumococcus had become resistant to the dog's immune bodies seems unlikely in view of studies on this point in other animals.

SUMMARY

A study was made of the changes in humoral immunity occurring during the early phases of experimental pneumococcus infection in the dog and cat. The methods devised by Robertson and Sia were employed to demonstrate the presence of anti-pneumococcus properties in the serum of animals naturally resistant to this micro-organism. It was found that with a generalized and overwhelming infection accompanied by early blood invasion, there was a prompt and rapid decrease in the concentration of natural humoral immune bodies which frequently disappeared entirely by the time of death. This same

early diminution of humoral immune substances, opsonins, agglutinins, and pneumococcidal-promoting bodies was observed in animals that survived a moderately severe generalized infection but the concentration of immune bodies rose again with the onset of recovery. The decrease in concentration of humoral immune substances during a severe generalized infection appeared to be due to the combination of "S" substance with the normal immune bodies.

When the pneumococcus infection was more localized as in the case of true lobar pneumonia a quite different sequence of events was observed to occur. Several animals, in which extensive lobar pneumonia was produced, showed the presence in quantity of humoral immune bodies in the blood throughout the course of an infection terminating fatally.

These findings suggest that after the inception of pneumococcus infection in the dog and cat the chief function of natural anti-pneumococcus substances in the blood is to limit or prevent blood invasion. When pneumococcic infection is localized these circulating antibodies appear to have little effect either in preventing the spread of the process or determining the outcome of the disease.

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