

STUDIES ON PNEUMOCOCCUS GROWTH INHIBITION.

V. THE RELATION OF VIRULENCE TO THE PNEUMOCOCCIDAL ACTIVITY OF NORMAL RABBIT SERUM-LEUCOCYTE MIXTURES.

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The present paper deals with a further study of the pneumococidal action of normal serum-leucocyte mixtures. Results of previous work (1), in which it was shown that the blood (serum and leucocytes) of certain pneumococcus-resistant animals possessed destructive properties for pneumococci, not found in the blood of susceptible animals, suggested that natural immunity to pneumococcus infection depends chiefly, if not entirely, on pneumococidal activity of the blood. In these studies, pneumococci of high virulence¹ only were employed, and the animals tested represented well marked examples of natural immunity and susceptibility, the dog and cat on one hand and the rabbit and guinea pig on the other. It is a very commonly observed fact that within a single species of any of the usual laboratory animals there occur wide variations in susceptibility toward different strains of disease-producing pneumococci, and that among the so called susceptible animals such variations may be extreme. But why rabbits, for example, should succumb to 0.000001 cc. of a culture of one strain of pneumococcus and resist successfully 0.1 cc. of another, has not been satisfactorily explained. The view that avirulence is to be accounted for by a failure of the microorganism to adapt itself to growth in the animal body does not seem to hold as a general explanation of this phenomenon, since pneumococci freshly isolated from cases of lobar pneumonia are found not infrequently to be of low pathogenicity for the rabbit and the guinea pig. Resistance against invasion by such

¹The word virulence is used here to indicate a high degree of invasiveness for the so called susceptible animals, rabbit, mouse, and to a less extent the guinea pig.

an organism would seem to be more than passive in nature. The following experiments were undertaken with the purpose of determining whether the immunity shown by a relatively susceptible animal, as the rabbit, against certain strains of pneumococci, is associated with demonstrable pneumococcus-destroying powers in the blood.

Earlier work on this subject consisted chiefly in testing the bactericidal properties of blood serum or plasma, alone or in the presence of leucocytes, against pneumococci of low virulence for the animal species. The ingredients of the test were mixed in the test-tube or capillary pipette, incubated, then cultured for surviving organisms. Results varied widely but it is generally agreed that these experiments failed to show conclusively the presence of pneumococcus-destroying properties in the blood. More recently Heist and Solis-Cohen (2) studied this question by means of seeding pneumococci of varying virulence for the rabbit into whole uncoagulated rabbit blood, which was then drawn up into capillary pipettes and incubated. They found that the ability of a given strain of pneumococci to grow in rabbit blood was directly proportional to the virulence of the strain, and furthermore, that the failure of avirulent organisms to grow was due to the pneumococidal action of the blood. However Bull and Bartual (3), in a series of carefully controlled experiments, were unable to confirm Heist and Solis-Cohen's findings. For a detailed discussion of the technique of these tests the reader is referred to the second paper of this series (1).

Technique.

Rabbits were employed throughout.² The growth inhibition tests were carried out as before (1). Pneumococci of Types I, II, II atypical, and III were tested. The rabbit leucocytes were obtained from an aleuronat pleural exudate on the day of experiment. Normal rabbit serum was procured from the animal supplying the leucocytes. These rabbits averaged 1500 to 1600 gm. in weight but occasionally larger ones were used.

For the virulence tests, young and actively growing cultures, with an opacity approximately equal to that of a "standard suspension" (4), were employed. Dilutions were made in meat infusion broth, pH 7.8. For each strain of pneumococcus a series of 3 to 4 rabbits was injected intraperitoneally with varying amounts of culture and observed for a period of 10 to 14 days. The rabbits were normal, full grown, and as nearly of the same weight as obtainable. When there were differences of weight in the series, the larger rabbits were given the larger doses of organisms. Repetition of these tests gave essentially the same results in all cases.

² Growth inhibition tests with the serum and leucocytes of guinea pig were found to be unsatisfactory for this purpose, apparently because of the greater susceptibility to injury of guinea pig leucocytes during washing.

EXPERIMENTAL.

Experiment 1.—(Table I.) Two types of pneumococcus of different virulence were used. The Pneumococcus Type I was originally isolated from a case of lobar pneumonia and kept in blood broth for 6 years with an occasional animal passage. 0.000001 cc. of culture killed rabbits of 1600 gm. in 24 hours. The Pneumococcus Type II was obtained from the Hospital of The Rockefeller Institute some months previously and is referred to as D39/21. After five passages

TABLE I.

Comparison of a Virulent Pneumococcus Type I with an Avirulent Type II.

Normal rabbit serum 0.2 cc. + rabbit leucocyte suspension 0.1 cc. + pneumococcus suspension 0.1 cc.

	Amount of standard suspension.	Color change at 24 hrs.	At 72 hrs.		Virulence test.
			Pneumococci in stained film.	Growth in culture.	
	cc.				cc.
Avirulent Pneumococcus Type II.	0.01	+	+		0.01
	0.001	0	+		
	0.0001	0	0	0	
	0.00001	0	0	0	
	0.000001	0	0	0	
Virulent Pneumococcus Type I.	0.01	+	+		0.000001
	0.001	+	+		
	0.0001	+	+		
	0.00001	+	+		
	0.000001	+	+		
Controls with serum only.					
Avirulent Type II.	0.0000001		+		
	0.0000001		+		
Virulent Type I.	0.0000001		+		
	0.0000001		+		

through rabbits, its killing dose was found to be 0.01 cc. of the standard culture for rabbits of about 1500 gm. Agitation of the small tubes was maintained at twenty-four revolutions per minute continuously for 24 hours. The tubes were then left in the incubator without agitation for another 48 hours, at the end of which time smears were made from their contents with aseptic precautions. Those tubes showing no organisms in stained smear were cultured in dextrose blood broth.

It will be seen in Table I that the growth of the Pneumococcus Type II of low virulence (for rabbits) was inhibited to a marked degree,

while the highly virulent Type I grew out in the smallest numbers used. Tests were next carried out to determine whether similar differences between virulent and avirulent organisms were to be found in other types and strains of pneumococci.

Experiment 2.—(Table II.) Two types of pneumococcus were used: a Type II of low virulence (employed in Experiment 1), and a Type II atypical of high

TABLE II.

Comparison of Virulent Pneumococcus Type II Atypical with an Avirulent Type II.

Normal rabbit serum 0.2 cc. + rabbit leucocyte suspension 0.1 cc. + pneumococcus suspension 0.1 cc.

	Amount of standard suspension.	Color change at 24 hrs.	At 72 hrs.		Virulence test.
			Pneumococci in stained film.	Growth in culture.	Minimum lethal dose of culture.
	cc.				cc.
Avirulent Pneumococcus Type II.	0.01	+	+		0.01
	0.001	0	+		
	0.0001	0	0	0	
	0.00001	0	0	0	
	0.000001	0	0	0	
	0.0000001	0	0	0	
Virulent Pneumococcus Type II atypical.	0.01	+	+		0.00001
	0.001	+	+		
	0.0001	+	+		
	0.00001	+	+		
	0.000001	+	+		
	0.0000001	+	+		
Controls with serum only.					
Avirulent Type II.	0.0000001		+		
	0.0000001		+		
Virulent Type II atypical.	0.0000001		+		
	0.0000001		+		

virulence for rabbits, obtained from the blood of a case of lobar pneumonia. Its minimum lethal dose was 0.00001 cc. of the standard culture for rabbits of 1350 gm. The test was carried out exactly as in Experiment 1.

The outcome of Experiment 2 (Table II) is similar to the finding in Experiment 1. The rabbit serum-leucocyte mixture failed to inhibit the growth of even a minute number of the virulent Type II atypical

pneumococcus but showed a marked bactericidal action for the strain possessing only a slight virulence for rabbits.

Growth inhibition tests were performed with a number of strains of Pneumococcus Type II, several of which had been isolated freshly from cases of lobar pneumonia. There was marked inhibition of growth in every instance and all these strains were found to be of low virulence for rabbits. On the contrary, all the Type I strains studied have been found to possess high virulence for rabbits and they grew readily in rabbit serum-leucocyte mixtures.

Tests performed with Type III pneumococci of varying virulence for rabbits gave, in general, the same results as were found with Types I and II, except that the bactericidal action of the serum-leucocyte mixtures for them was not as marked; *i.e.*, given a Type II and a Type III of about equally low virulence, the growth of Type II was inhibited to a considerably greater degree than was that of Type III. Consequently the difference between the virulent and the avirulent Type III organisms as tested in this way was less than that observed in the above experiments with Types I and II.

It was then considered desirable to determine whether enhancement of virulence by animal passage would result in a corresponding increase in the ability of the organism to grow in rabbit serum-leucocyte mixtures. Accordingly, attempts were made to increase the virulence of several stock strains of Types II and III, including those used in the foregoing experiments, but without success. A long series of animal passages in rapid succession was employed. In certain cases passage through mice and guinea pigs as well as rabbits was resorted to. Direct injection of heart's blood was also used. But at the end of 20 to 40 passages, the virulence for rabbits of the several strains was found to be only slightly increased. Comparative growth inhibition tests on these strains before and after animal passage showed no essential difference between them. This again strongly suggests that the pneumococidal properties of the rabbit's blood reveals the state of the animal's resistance to the pneumococcus in question.

Another means of determining the relation between the pneumococidal properties of the blood and susceptibility to pneumococcus infection was to be found in comparing the serum-leucocyte mixtures of full grown and young rabbits, since the latter are much more

susceptible to pneumococcus infection. It was ascertained that certain strains of Pneumococcus Type II, relatively avirulent for adult rabbits, killed young rabbits in minute doses. In the following experiment the growth ability of such a Type II was tested in the serum and leucocytes of a large and a small rabbit respectively.

TABLE III.

Comparison of Serum-Leucocyte Mixtures of the Full Grown and Young Rabbit.

Normal rabbit serum 0.2 cc. + rabbit leucocyte suspension 0.1 cc. + pneumococcus suspension 0.1 cc.

	Amount of standard suspension.	Color change at 24 hrs.	At 72 hrs.		Virulence test.
			Pneumococci in stained film.	Growth in culture.	Minimum lethal dose of culture.
	cc.				cc.
Serum and leucocytes of full grown rabbit (1650 gm.).	0.01	+	+		
	0.001	0	0	0	
	0.0001	0	0	0	
	0.00001	0	0	0	0.01
	0.000001	0	0	0	
	0.0000001	0	0	0	
Serum and leucocytes of young rabbit (620 gm.).	0.01	+	+		
	0.001	+	+		
	0.0001	+	+		
	0.00001	0	+		0.00001
	0.000001	0	+		
	0.0000001	0	+		
Controls with serum only. Full grown rabbit.	0.0000001		+		
	0.0000001		+		
Young rabbit.	0.0000001		+		
	0.0000001		+		

Experiment 3.—(Table III.) The Pneumococcus Type II used was the same strain employed in Experiment 1. Its minimum lethal dose was 0.01 cc. of the standard culture for rabbits of 1500 gm. and 0.00001 cc. for rabbits of 650 gm. Leucocytes and serum were obtained from a large rabbit weighing 1650 gm. and a small rabbit weighing 620 gm. Two tests were set up at the same time. The technique of the test was as before.

The difference between the pneumococcal activity of serum and leucocytes of large and small rabbits is strikingly shown in Table III.

While quantities of the standard suspension less than 0.01 cc. grew out in the adult rabbit serum-leucocyte mixtures, 0.0000001 cc. grew out in the serum and leucocytes of the young rabbit. Thus the development of pneumococidal power in the blood appears to accompany the increased resistance to pneumococcus infection shown by the full grown rabbit. While complete studies of the parallelism between the acquisition of pneumococcus immunity and blood pneumococidal activity by the growing rabbit have not been made, it has been found that the half grown rabbit of 1000 gm. in weight possesses both these properties to a considerable degree but not as marked as in the adult animal.

DISCUSSION.

The results of the above experiments suggest that the more susceptible animals, of which the rabbit is a type, possess the same kind of defense mechanism against pneumococcus infection as do the highly pneumococcus-resistant animals; *e.g.*, dog and cat. In examples of both classes of animal the blood has been shown to exert a marked destructive action on pneumococci of low virulence for the species studied. However, the differences in blood pneumococidal power, between these animals so widely separated in degree of susceptibility to the highly virulent strains of pneumococci, are pronounced. This is shown by the fact that the serum-leucocyte mixtures of the dog or cat are capable of destroying virulent pneumococci in large numbers, whereas the serum and leucocytes of the rabbit are found to be without bactericidal activity for them. But whether the greater potency of the blood elements of the highly resistant animal is due to a higher concentration of the same pneumococcus-destroying properties occurring in the blood of the relatively susceptible animal, or is due to some added element, the foregoing experiments do not make clear. An analysis of this reaction will be dealt with in a subsequent communication.

The absence of pneumococidal properties in the blood of the very young rabbit and the development of such properties during growth, would seem to account for the marked resistance shown by the adult rabbit against pneumococci of high fatality for the young animal. There may be other unknown factors, such as possible differences in

susceptibility of adult and young tissue cells to the products of pneumococcus growth, which act in conjunction with the pneumococcus-destroying powers of the blood as codeterminants of susceptibility and resistance. Yet even if such accessory factors do play a part, the ability of the body to destroy pneumococci is probably still of chief importance in preventing infection.

The direct parallelism between virulence for rabbits and ability to grow in rabbit serum-leucocyte mixtures, points to the possession by the virulent pneumococcus of a specific property capable of neutralizing the pneumococidal action of the serum and leucocytes. There is reason to assume also that it is the lack of this same property, or its possession to a much less degree, which renders the avirulent pneumococcus susceptible to destruction by serum-leucocyte action, since the culture medium provided by the fresh serum alone, or by leucocytes and inactivated serum, is an excellent one for the growth of such organisms. These findings help to narrow the study of pneumococcus virulence to an investigation of the different reactions which may occur between the pneumococcus and its products, and the serum and leucocytes. The results of further observations of this nature are presented in the following paper.

SUMMARY.

Employing a method, described in an earlier publication, for testing the pneumococidal activity of serum-leucocyte mixtures, a study has been made of the pneumococcus-destroying properties of the blood of a relatively susceptible animal, the rabbit, for pneumococci of low virulence for the species. It was found that rabbit serum-leucocyte mixtures possessed the power to kill avirulent pneumococci in relatively large numbers but failed to inhibit the growth of virulent organisms even in minute quantities. The results of numerous experiments in which all three types of pneumococci were employed indicated that the ability of a strain of pneumococcus to grow in rabbit blood is dependent on its virulence for the rabbit. The extreme susceptibility of the very young rabbit to strains of pneumococcus of low virulence for the full grown animal, was found to be associated with an absence of pneumococidal properties in the blood of the young rabbit. These findings suggest that the relatively susceptible animals

possess the same type of defense mechanism against pneumococcus infection as do the highly pneumococcus-resistant species.

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