STUDIES ON IMMUNITY TO PNEUMOCOCCUS MUCOSUS (TYPE III).

II. The Infectivity of Type III Pneumococcus for Rabbits.

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In a previous paper (1) it was shown, in agreement with the experience of others, that the sera of rabbits immunized with Type III pneumococci failed to agglutinate the homologous organisms. Evidence was offered that the failure in agglutination was due, not to the inagglutinable state of the encapsulated bacteria, but to the actual absence of demonstrable type-specific antibodies. However, the sera of the immunized animals were reactive with pneumococcus nucleoprotein regardless of its type derivation and were capable of agglutinating the non-encapsulated degraded variants of all type-specific strains (R forms). This antibody response is contrary to the usual experience in immunization with type-specific pneumococci, when the cells used as antigen are intact. Although the Type III organisms used were, for the most part, possessed of large capsules, the antibody response, instead of being type-specific, was only species-specific in character and simulated that elicited by immunization with pneumococcus protein or with non-encapsulated R forms. The inference drawn from these results was that rabbits possess some mechanism which is capable of affecting the antigenic integrity of Type III pneumococci and that the alteration which the organisms undergo in the animal body is reflected in the character of the antibody response.

Because of the unusual reaction of rabbits to immunization with Type III pneumococci and the implications which these results suggest, investigations have been carried out with regard to the infectivity of this type of pneumococcus for rabbits. The experiments reported in the present paper include observations on the degree of viru-

lence of several strains of Type III, on the character of the bacteremia following intravenous injection, and the relation of phagocytosis to the disposal of the injected organisms.

Although Type III pneumococci are recognized as being highly pathogenic for white mice, reports on their virulence for rabbits have differed. Hanes (2) states that Type III organisms are highly virulent for rabbits without giving dosage or number of strains tested. Singer and Adler (3) found 0.05 to 0.1 cc. of culture usually fatal, but mention the fact that the lethal dose, in their experience, was not constant. They employed two strains and found one more constantly virulent than the other. Lévy-Bruhl (4) found the minimum lethal dose to be 1 cc. with two strains of Type III and greater than 1 cc. with two other strains. Bengtson (5) reported the lethal dose to vary from 0.1 to 1 cc.; whether different strains were tested or not is not stated.

In order to obtain further information concerning the virulence of Type III pneumococci for rabbits, eleven strains of this organism were collected. The strains were obtained from either blood or sputum of patients suffering from lobar pneumonia. All of the strains, which had been recently isolated were found on first injection to be highly virulent for mice. Other strains, which were taken from stock, were first passed through mice to enhance their virulence for these animals before being tested in rabbits. This was done in order to exclude from the cultures degraded R forms of pneumococci, which are non-encapsulated, non-type-specific, and avirulent. Reimann (6) has pointed out that a pneumococcus culture of low virulence may be one in which R forms predominate over type-specific, encapsulated S forms. According to this view, the repeated passage through mice of a culture containing both forms increases the proportion of S organisms until, with the acquisition of maximum virulence, the culture is theoretically composed entirely of type-specific, encapsulated pneumococci. Consequently, the cultures of Type III used for virulence tests in rabbits, by first being made highly pathogenic for mice, fulfilled this requirement.

Rabbits were injected either intravenously or intraperitoneally, with 12 to 14 hour cultures in doses ranging from 2 to 10 cc. It may be seen from Table I that eight of the strains did not produce fatal infection. In the case of two other strains rabbits died following injection of relatively large doses, but the cultures isolated from the

blood of these failed to kill others. Although no sustained effort was made to enhance the virulence of all the strains, most of them were injected several times. Of the eleven strains only one acquired definite virulence for rabbits, which, by repeated passage, finally produced a fatal infection in doses of 0.0001 cc. Even with this strain

TABLE I.

Virulence of Type III Pneumococcus for Rabbits.

Strain of Type III	Virulence for mice	Rabbit No.	Dose of culture	Site of injection	Results
	cc.		*cc.*		
A	.000001	1	5	Intravenous	Survived
A	.000001	2	2	Intraperitoneal	"
A	.000001	3	2	"	Died—2 days
Blood culture Rabbit 3		4	2	"	Survived
" " "		5	5	"	"
M	.000001	6	2	Intravenous	"
M	.000001	7	3	Intraperitoneal	"
M	.000001	8	10	"	"
F	.000001	9	5	"	Died—3 days
Blood culture Rabbit 9		10	5	"	Survived
F	.000001	11	8	"	"
L	.000001	12	2	Intravenous	"
L	.000001	13	5	Intraperitoneal	"
H	.000001	14	5	Intravenous	"
H	.000001	15	10	Intraperitoneal	"
E	.000001	16	5	"	"
S	.000001	17	5	"	"
S	.000001	18	8	"	"
B3	.000001	19	5	"	"
B3	.000001	20	2	Intravenous	"
B4	.000001	21	5	"	"
B4	.000001	22	5	Intraperitoneal	"
B2	.000001	23	5	"	"
PH*	.000001	24	5	"	Died—2 days

* This strain became highly virulent for rabbits.

the degree of virulence has not remained constant and has often shown evidence of attenuation when kept out of the animal body for several days. Moreover, differences in the natural resistance of individual rabbits to the same strain is, in part, responsible for variations in the degree of virulence.

From the results obtained it seems justifiable to conclude that Type III pneumococci possess only slight initial virulence for rabbits. Furthermore, although type specificity and encapsulation are necessary for the highest degree of mouse virulence, these characteristics are not, in themselves, sufficient to overcome the natural resistance possessed by rabbits.

The conclusion that Type III pneumococci are of low pathogenicity for rabbits was arrived at by the simple procedure of injecting organisms and accepting the ultimate survival or death of the animal as a criterion of virulence. This method, however, throws no light either on the duration and intensity of the infection, or upon the method of recovery, a fact which has been emphasized by Bull (7) in a report on some of the characteristics of streptococcal and pneumococcal bacteremia in rabbits. The technique employed by him consisted in making blood cultures intermittently from the peripheral veins after the injection of organisms. The results obtained by Bull and others (7–10) have demonstrated the reliability of the method. Consequently, since Type III pneumococci failed to produce fatal infections in rabbits, it seemed of interest to observe the course of the bacteremia following injection of these organisms. This was done by means of blood cultures taken at frequent intervals following the introduction of organisms into the circulation. The results are diagrammatically represented in the accompanying text-figures in which the number of colonies per unit of blood is plotted on the ordinates and the time interval, at which the culture was taken, is plotted on the abscissæ (Text-figs. 1, 2, and 3).

There were available for this study both S and R strains of Type III pneumococci. The eleven S strains were typical and biologically identical. They possessed large, easily demonstrable capsules; they grew on blood agar with the production of mucoid colonies; they were bile-soluble; they all reacted equally well in Type III antipneumococcus horse serum. They were pathogenic for humans, the source from which they were derived, and were all equally virulent for mice, killing in doses of 0.000001 cc. However, one of the S strains differed in that it was made virulent for rabbits by rabbit passage, whereas the others were not virulent for these animals in doses ranging from 2 to 10 cc. In addition to the type-specific S strains of pneumococci, non-

type-specific, degraded R forms were used for comparative study. As described by Reimann (6) R forms may be obtained by cultivating type-specific organisms in homologous immune serum. Those used in this experiment were derived from a culture of Type III. Doses of 1 cc. failed to kill mice and doses of 10 cc. failed to kill rabbits; higher doses were not tested. The strains of pneumococci used in the present experiment, then, comprised representatives of each of the three varieties:

1. S strains of Type III pneumococcus; *virulent* for rabbits (designated SV). One strain belonged to this group.

2. S strains of Type III pneumococcus; *avirulent* for rabbits (designated SA). Ten strains belonged to this group.

3. R strains representing the degraded, non-encapsulated, avirulent variants of Type III pneumococci. Bacteria of this character are comparable to non-pathogenic saprophytes.

EXPERIMENTAL.

Cultures.—12 to 14 hour plain broth cultures in standard doses of 2 cc. each were injected intravenously regardless of the strain used. The actual number of organisms per cc. was not determined but under uniform conditions, it may be considered comparable for all the strains.

Blood Culture Technique.—The technique employed varied only in minor details from that described by Bull (7) and was as follows: The organisms were introduced into the marginal vein of one ear of the rabbit and cultures were obtained from the opposite ear. Before making the culture, the ear was closely shaved along the marginal vein and then saturated with 95 per cent alcohol. The alcohol was wiped off with a dry sterile sponge and the vein slit transversely with a razor blade. The blood was allowed to drip perpendicularly from the edge of the ear and, after discarding the first few drops, 6 drops were collected in melted agar kept at 42° to 45°C. The melted agar and blood were mixed rapidly and poured into a Petri dish. The plates were incubated for 36 hours and the number of colonies per plate recorded. Cultures were usually taken 15, 30, and 60 minutes after injection of the organisms, then at 2 hour intervals for 12 hours; after this, 2 or 3 times daily until the animal succumbed or permanent sterility occurred. After the first 12 hours subsequent cultures were taken from blood derived from a fresh slit distal to the previous cuts. This precaution was taken in order to obviate the possibility of organisms resident in the tissues at the site of the previous injury being washed into the agar by the flowing blood.

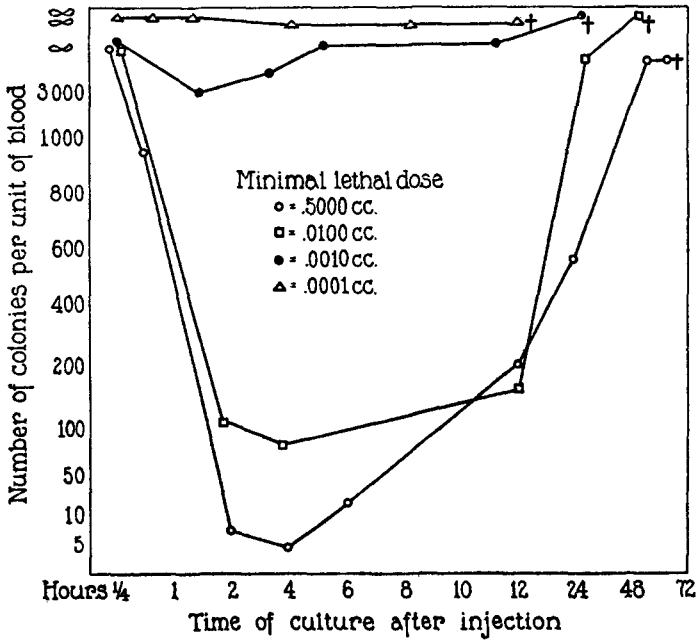
Employing streptococci and pneumococci Bull (7) found that infection in rabbits may take one of three courses depending on the viru-

lence of the bacteria. With highly virulent organisms a rapidly fatal septicemia occurred which was characterized by an initial marked reduction in the number of organisms in the circulating blood, followed by a rapid increase. With less virulent organisms the infection became chronic, the course of the bacteremia was uneven, and, after death, localization of organisms in the serous cavities was often demonstrable. When bacteria of even lower virulence were injected, they quickly disappeared and the blood stream remained sterile. The initial diminution in the number of organisms occurring in experimental septicemia, first described by von Fodor (8), has been repeatedly observed by others. This phenomenon has usually been explained as being due to the dissemination and filtration of the organisms throughout the body tissues. However, that this explanation is not sufficient becomes evident, as will be shown, if the relative virulence of the bacteria injected is taken into consideration.

Course of Bacteremia Following Intravenous Injection of Rabbit Virulent, S Strain of Type III Pneumococcus (SV Strain).

The effect of increased virulence on the course of the bacteremia is shown in Text-fig. 1. These results were obtained by injecting rabbits with a strain of Type III pneumococcus, the virulence of which had been progressively increased by repeated passages through rabbits. The data presented reveal differences in the course of the bacteremia depending on the degree of virulence. When the minimal lethal dose of the rabbit virulent strain (SV) was 0.5 cc. there was a marked initial decrease in the number of organisms in the circulating blood, the minimum occurring 4 hours after infection; then there followed a progressive rise in the number of colonies until the death of the animal occurred 56 hours after injection. This type of curve in experimental septicemia corresponds to the results previously described by others. When the virulence of this same strain had been enhanced so that now, 0.01 cc. of culture proved fatal, the initial decrease was less marked, and the secondary rise more rapid. Injection of a culture of still greater virulence (0.001 cc.) resulted in only a slight initial decrease in the number of organisms in the peripheral blood. When a maximum virulence of 0.0001 cc. had been attained the number of organisms in the blood of the infected rabbit was at no

time decreased and death resulted in 12 hours. In each instance approximately the same number of organisms was injected. Therefore, if the primary decrease represented merely a mechanical process of dissemination and filtration it should occur regularly, regardless of virulence. This, however, was not the case; the extent of the initial decrease in the number of circulating bacteria was in inverse proportion to the degree of virulence (Text-fig. 1).

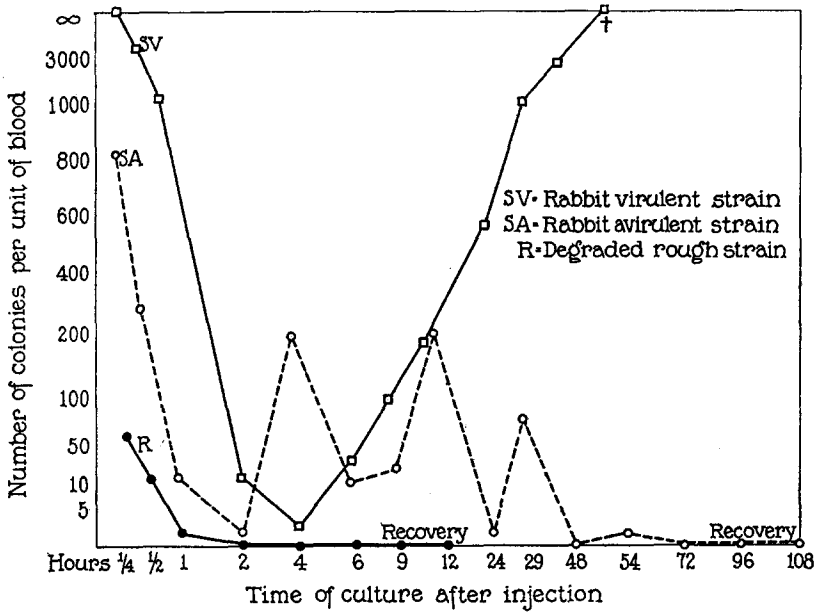


TEXT-FIG. 1. Effect of increased virulence on the course of bacteremia in rabbits injected with Pneumococcus Type III (Strain SV).

In addition to the effect of progressively increased virulence on the course of blood infection caused by the rabbit virulent strain of pneumococcus, certain characteristic differences in the curves were observed when strains of Type III not virulent for rabbits and degraded R forms of pneumococcus were employed. A graphic representation of the course of the bacteremia following injection of organisms of these two varieties is shown in Text-fig. 2. For purposes of comparison the type of curve representing the course of events after injection of the rabbit virulent strain is included in this same experiment.

Course of Bacteremia Following Intravenous Injection of R Strains of Pneumococcus.

From Text-fig. 2 it may be seen that there was an immediate and marked decrease in the number of R organisms in the circulating blood. After 2 hours they completely disappeared and the rabbit remained free from further blood infection.

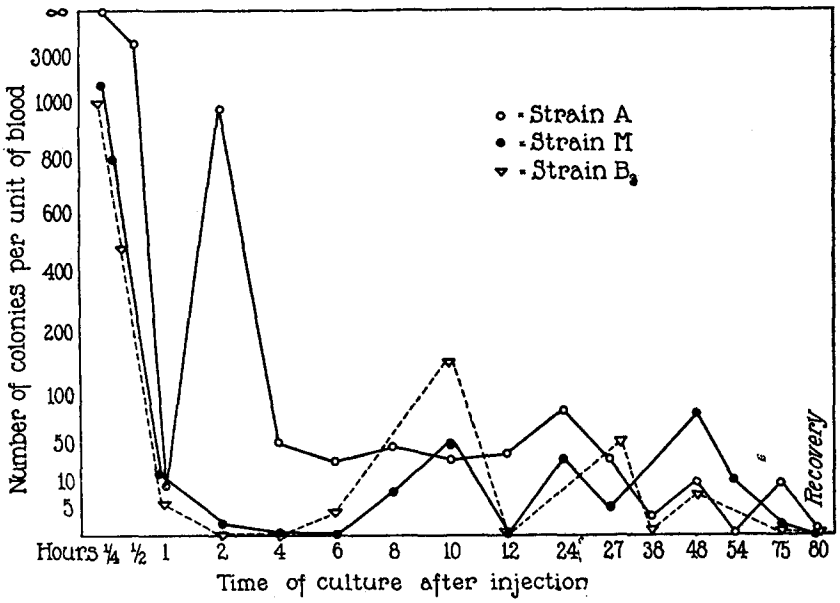


TEXT-FIG. 2. Course of bacteremia in rabbits injected with Pneumococcus Type III (Strains SV, SA, R).

Course of Bacteremia Following Intravenous Injection of Rabbit Avirulent, S Strains of Type III Pneumococcus (SA Strains).

The bacteremia which followed injection of rabbit avirulent strains of Type III pneumococci ran a distinctly different course. From Text-fig. 2 it may be seen that 3 to 4 days elapsed before permanent sterility of the blood was finally attained. While the bacteremia persisted, the chief characteristic was repeated fluctuations in the number of organisms in the circulating blood. At times the cocci entirely disappeared only to reappear again a few hours later. Exami-

nation of the organisms which reappeared failed to reveal any biological differences, when compared with the original cultures. As previously stated, the rabbit avirulent strains (SA) represent all but one of those used in the experiments. Although only one curve characteristic of the bacteremia produced by these strains is shown in Text-fig. 2, repetitions of this experiment gave similar results. In Text-fig. 3 are shown similar curves obtained with three other rabbit avirulent strains.



TEXT-FIG. 3. Course of bacteremia in rabbits injected with strains of Pneumococcus Type III, avirulent for rabbits (Strains SA).

In the experiments just described virulence of Type III pneumococci for rabbits has been considered with special reference to certain properties pertaining to the organisms themselves, such as differences of encapsulation, type specificity, and mouse virulence. These factors have been correlated with the infectivity of the organisms as represented, not only by the ultimate outcome of the infection in rabbits, but also by the character of the bacteremia produced.

In addition to the biological properties of the bacteria, factors related to the resistance of the host require consideration and an investigation of two of these has been carried out.

1. Antibodies.—The sera of normal rabbits have not been found to possess antibodies reactive with the encapsulated Type III cells, or with the soluble specific substance derived from these organisms, or to confer passive protection on mice. Although normal rabbit sera are occasionally encountered which agglutinate R pneumococci, the incidence is not common and the dilutions of serum in which the reaction occurs are low. Whether antibodies of this character are significant in the disposal of R cells after injection into the animal body, is irrelevant to the present study. It can be stated, however, that the phagocytosis of R pneumococci, as described below, is not preceded by agglutination.

2. Phagocytosis.—In studying phagocytosis, the vital staining method described by Sabin (11) for the study of living blood cells has been employed. The technique varied in no detail from Sabin's procedure except for the fact that a small loopful of living pneumococcus culture was added to the blood. The preparations were observed microscopically in a warm chamber kept at 37°C. By this method leucocytes may be watched immediately after removal from the animal body and while actively motile in the environment of whole blood. Preparations of this character approximate conditions existing in the circulating blood and minimize alterations of the leucocytes which may occur in the usual methods of studying phagocytosis *in vitro*. The specimens were observed from 1 to 2 hours. At the end of this time the leucocytes begin to lose their motility and their protoplasm contains large, red staining bodies which Sabin has described as vacuoles. Observations beyond 1 to 2 hours have not revealed phagocytosis which was not present earlier, and the beginning alteration in the leucocytes was evidence that the preparations were not useful for further observation.

When R forms of pneumococci were employed, phagocytosis by polymorphonuclear leucocytes could be seen in 2 to 3 minutes. It continued actively and, after 10 minutes, every leucocyte was engorged with organisms. When phagocytosis is observed under these conditions of vital staining the picture is very striking. Pneumococci, before ingestion, are unstained. However, immediately after being engulfed, they appear as bright red organisms within the body of the leucocyte. The facility with which R cells are phagocyted is

evidence of the prime importance of this activity in the natural resistance of rabbits to blood infection with R forms; the promptness with which these organisms disappear from the blood stream following intravenous injection may be referable, in part at least, to their susceptibility to phagocytosis.

When S forms of Type III pneumococci were mixed with normal rabbit blood, no phagocytosis was observed. This was true whether the encapsulated organisms were virulent or avirulent for rabbits. Occasionally, after an hour a few leucocytes could be found which had ingested one or two bacteria but the picture contrasted sharply with that seen in preparations made with R forms. Since the inability of leucocytes to ingest encapsulated bacteria has been a common observation, and, since this fact has been related to virulence, the failure to demonstrate this correlation in the present study is of special significance. Although phagocytosis as observed *in vitro* may not be identical with the phenomenon *in vivo*, nevertheless, the contrast between the action of leucocytes against R and S forms is striking and justifies the inference that the natural resistance of rabbits to S forms of Type III pneumococci involves factors either additional to or different from phagocytosis.

DISCUSSION.

In the experiments reported in this paper on the infectivity of Type III pneumococci for rabbits certain factors which are known to be associated with virulence and resistance have been taken into consideration. The organisms used in the experiments conformed with certain requirements which might be expected to promote virulence. All of the strains possessed large mucoid capsules and type specificity (S forms). They were obtained from patients suffering from lobar pneumonia and were highly virulent for mice. However, in spite of this, ten out of eleven of these strains failed to produce fatal infection in rabbits when injected in moderately large doses. The other strain was slightly virulent for rabbits on isolation and this property was further enhanced by animal passage. Since no biological differences could be demonstrated between this strain, when possessed of maximum virulence, and the others, it is necessary to assume that viru-

lence in this instance is related to some property not possessed by the other S strains. The course of the fatal septicemia resulting from injection of this strain and alterations in the character of the curve associated with increased virulence are shown in Text-fig. 1.

An attempt to understand the failure of *avirulent, encapsulated* Type III pneumococci (S forms) to cause fatal infection in rabbits led to a study of the fate of these organisms as contrasted with that of *avirulent, non-encapsulated* pneumococci (R forms). Although the ultimate survival of the infected animal occurs in both instances, the bacteremia induced by S forms differs from that induced by R forms. Following injection, the non-encapsulated R cells disappear rapidly and permanently from the blood stream, whereas the avirulent, but encapsulated S forms give rise to a prolonged bacteremia characterized by intermittent increase and reduction in the number of circulating organisms. These differences suggest that the mechanism whereby rabbits overcome the two types of infection is not identical, and that the method of disposal is in some way related to the presence or absence of capsule. Further evidence that the mechanism of recovery in the case of S organisms is different from that effective in disposing of R forms, was brought out in the experiments on phagocytosis. Therefore, it seems obvious that, although the encapsulated state is sufficient to prevent phagocytosis, it is not sufficient to protect the cell against the defense reactions of the host. The resistance of rabbits to Type III pneumococci implies the presence of factors other than phagocytosis. Furthermore, the fact that the sera of normal rabbits do not contain demonstrable type-specific antibodies renders improbable the participation of these immune substances in the mechanism of natural resistance. In a previous paper (1) it was shown that immunization of rabbits with Type III pneumococci failed, in the majority of instances, to stimulate the production of type-specific antibodies, but was effective in producing antibodies reactive against another constituent of pneumococci, namely the nucleoprotein fraction. These experiments indicate that normal rabbits possess a mechanism which is capable of inflicting an injury on the capsule of Type III pneumococci. It has been shown by Avery and Heidelberger (12) that type-specific antibodies are best elicited when S cells in an intact state are used as antigen. They (12) have also

shown that type specificity is intimately related to the soluble specific substance of the capsule of pneumococcus. Therefore, the absence of demonstrable type-specific antibodies in rabbits immunized with encapsulated Type III pneumococci indicates that the animals are capable of damaging the capsular mechanism of these organisms. In the present experiments, the capacity of normal rabbits to inflict injury on Type III pneumococci is further demonstrated by the recovery of the animals following intravenous injection of living S organisms. It seems not unlikely that both the survival of the animal and the altered antigenicity of the cell following injection of the encapsulated organisms are referable to the same mechanism, and upon the factors underlying this defense reaction depends the natural resistance of rabbits to Type III pneumococci.

SUMMARY.

The observations recorded in this paper on the infectivity of Type III pneumococci for rabbits may be summarized as follows:

1. Of eleven strains of Type III isolated from human sources, ten were found to possess low virulence for rabbits. This was true despite the fact that all the strains tested possessed large capsules and a high degree of virulence for mice.

2. One strain of Type III pneumococcus was rendered highly virulent for rabbits. Since it possessed no other biological property demonstrably different from the other strains, its virulence must reside in some additional property.

3. An initial decrease in the number of circulating organisms following the injection of virulent bacteria is a well known occurrence, and it was observed in rabbits injected with the rabbit virulent strain of Type III. However, the extent of the reduction was in inverse proportion to the degree of virulence of the strain; a fact which makes mechanical explanations of the phenomenon insufficient.

4. The bacteremia produced in rabbits by Type III pneumococci, avirulent for this species, runs a characteristic course. It differs from that produced by non-encapsulated R forms of pneumococci although in both instances survival of the infected animal ensues. This is evidence that the mechanism of resistance against encapsulated and non-encapsulated pneumococci is not identical.

5. Phagocytosis of Type III pneumococci by circulating rabbit leucocytes was not demonstrable by a vital stain technique, whereas under the same conditions the ingestion of non-encapsulated R forms occurred. This is further evidence that the process whereby non-encapsulated pneumococci are disposed of, is insufficient to explain the natural resistance of rabbits to infection with encapsulated Type III pneumococci.

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