STUDIES ON IMMUNITY TO PNEUMOCOCCUS MUCOSUS (TYPE III).

III. Increased Resistance to Type III Infection Induced in Rabbits by Immunization with R and S Forms of Pneumococcus.

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In two preceding papers (1, 2) certain phenomena concerning the antigenicity and infectivity of Type III pneumococci in rabbits were described. The results of these experiments may be summarized as follows: Immunization of rabbits with Pneumococcus Type III (1) fails, in the great majority of instances, to stimulate the production of type-specific antibodies, but is always effective in eliciting antibodies reactive with pneumococcus nucleoprotein and R strains derived from all types of pneumococci. These results were interpreted as indicating that normal rabbits possess some mechanism whereby, following the introduction of Type III organisms into the animal body, the antigenic complex of the bacterial cell is so altered that the type-specific component is rendered ineffective as antigen. Since Avery and Heidelberger (3) have shown that type specificity resides in the soluble specific substance which is predominantly present in the capsule, it appears that this altered antigenicity is the result of an injury inflicted upon the capsular substance of the cell. Further evidence in support of this view lies in the fact that Pneumococcus Type III possesses low pathogenicity for rabbits. Ten out of eleven strains of this organism, although highly pathogenic for white mice and possessed of large mucoid capsules (S forms), were found to be avirulent for rabbits in doses of 2 to 5 cc. and sometimes 10 cc. (2). Since encapsulation and virulence are generally considered as being intimately associated, it seems possible that the method, whereby

rabbits resist Type III infection, rests on the same mechanism which is responsible for the destruction of the type-specific antigenicity of the cell and that by virtue of the injury inflicted on the capsule of the living organism, virulence is impaired. These results expressed in terms of the host imply that rabbits possess a considerable degree of natural resistance to Type III infection. However, to attribute resistance to natural immunity is merely restating the problem and leaves unexplained the mechanism in the body on which it depends. In the recovery of normal rabbits from Type III infection the evidence indicates that in this instance, the factors underlying resistance are primarily operative against the capsular component of the cell.

The results previously referred to have concerned the reaction of normal rabbits. The experiments reported in this communication deal with immunized rabbits, and the purpose has been to determine the presence of active immunity against Type III infection following immunization not only with homologous organisms but also with heterologous strains, both S and R forms, and with solutions of pneumococci. Others (7, 8) have reported that rabbits immunized with Pneumococcus Type III are actively immune against infection with homologous organisms even though specific agglutinins are not demonstrable. These results minimize the rôle which specific antibodies play in active resistance in the case of Type III infection, and Singer and Adler (8) have offered an explanation of this phenomenon to which subsequent reference will be made. It seemed of significance to determine if any demonstrable antibody participated in this active immunity; consequently the sera of all the experimental animals have been tested for both type-specific (anti-S), and species-specific (anti-P), antibodies and the results correlated with the presence or absence of effective resistance.

Testing for the presence of active immunity necessitates the use of virulent organisms. Although most of the Type III strains have been found to possess low initial virulence for normal rabbits, one strain was made highly pathogenic by rapid animal passage. Consequently this rabbit virulent strain afforded a means of testing for increased resistance. Its virulence was maintained so that .001 cc. always proved fatal and, in most of the experiments, .0001 cc. caused death. Since a standard dose of 1 cc. of culture was always injected,

each animal may be considered as receiving usually 10,000 and always 1000 lethal doses.

Methods.

Antigens.—Heat-killed cultures, regardless of the type or strain employed in immunization, were, in each instance, made by the same method. 12 to 14 hour plain broth cultures, killed by heating at 56° for ½ hour, were centrifuged and resuspended in physiological salt solution in such quantities that 0.5 cc. of the vaccine was equivalent to 1 cc. of original culture.

The pneumococcus solutions used for immunization were made by two different methods. One solution consisted of nucleoprotein derived from Type II pneumococcus according to the method described by Avery and Morgan (4). The other solution was made as follows: 6 liters of R_2 culture (derived from Type II pneumococcus) were centrifuged and the bacteria resuspended in 100 cc. of physiological salt solution. 0.3 cc. of 10 per cent sodium desoxycholate was added, and the mixture incubated at 37.5°C. for 2 hours. At the end of this time stained films of the fluid showed a complete dissolution of all formed cells. The solution was then centrifuged at high speed to remove detritus, and the supernatant fluid filtered through a Berkefeld V filter. The bacteria-free filtrate was used for immunization.

Methods of Immunization.—All rabbits receiving heat-killed organisms were immunized according to the method described by Cole and Moore (5), which consists in alternating for 6 weeks, a week of daily injections of 0.5 cc. of vaccine followed by a week of rest.

Rabbits injected with pneumococcus solutions received 0.5 cc. intravenously each day for the 1st week and during the 2 other alternate weeks of injection received 1 cc. daily.

All animals were bled 8 to 10 days after the last injection and the antibody content of the sera determined.

Method of Testing Active Immunity.—As previously stated, a standard dose of 1 cc. of culture of the rabbit virulent strain of Type III was intravenously injected in each test animal. .0001 cc. of this culture was usually fatal and .001 cc. always killed normal rabbits.

For purposes of following the course of the blood infection in both immunized animals and normal controls, blood cultures were taken at frequent intervals according to the method previously used (2). 4 to 6 rabbits in addition to 1 or 2 controls were tested simultaneously.

Most of the animals which died were examined post mortem with special reference to gross pathological changes in the serous cavities.

Altogether, 44 rabbits have been tested for the presence of active immunity against infection with a rabbit virulent strain of Pneumo-

coccus Type III. In addition to animals which, by surviving, demonstrated a solid immunity, others were considered as partially immune, which, although ultimately succumbing, showed evidence of increased resistance by reason of the duration of life, the character of the bacteremia as contrasted with controls, and the presence of localized infection found post mortem. A more detailed consideration of these factors will be given in the analysis of the results.

The experimental animals, depending upon the pneumococcus material used for immunization, may be grouped as follows:

I. Rabbits immunized with Pneumococcus, Type III.
II. Rabbits immunized with Pneumococcus, Type I or II.
III. Rabbits immunized with Pneumococcus, R strains.
IV. Rabbits immunized with solutions of Pneumococcus.

Group I. Rabbits Immunized with Type III Pneumococcus.

Twelve rabbits belong to this group. They were chosen from the 28 rabbits used in the immunization experiments previously reported (1). The strain of Type III used for immunization of these animals, although encapsulated, type-specific, and highly pathogenic for mice, was avirulent for rabbits in doses of 5 cc. 3 of the 12 possessed type-specific agglutinins in low titre (2 of the sera were not reactive beyond 1:2 dilution and the other was not reactive beyond 1:20 dilution). The sera of the other 9 contained no demonstrable type-specific antibodies. The sera of all the animals possessed antiprotein antibodies, and agglutinated R strains in 1:160 or 1:320 dilutions of serum.

Of the 3 rabbits whose sera possessed demonstrable type-specific antibodies, 1 survived, 1 lived 7 days and showed evidence of increased resistance, 1 died simultaneously with the control. Of the other 9 rabbits, 5 survived, 3 lived 11, 16, and 22 days respectively showing evidence of increased resistance, 1 died with the control.

In summary, it may be seen that of the 12 rabbits immunized with Type III pneumococcus, 6 survived, 4 showed evidence of increased resistance, and 2 died at the same time as the controls. These results expressed in percentage are: 50 per cent survived and, by including the partially immune, 83 per cent showed definite evidence of increased resistance.

Group II. Rabbits Immunized with Type I or Type II Pneumococci.

Fifteen rabbits belong to this group; 10 were immunized with Type II, 5 with Type I, and all were subsequently infected with Type III. The sera of these animals contained homologous type-specific agglutinins to the usual degree and also antiprotein antibodies agglutinating R pneumococci to approximately 1:160 dilution of serum. Of the 10 rabbits immunized with Type II, 7 survived, 2 living 6 and 7 days respectively, were considered as possessing some immunity, 1 was unprotected. Of the 5 rabbits immunized with Type I, 4 survived and 1 was unprotected. The results obtained with rabbits immunized with heterologous type-specific pneumococci may be summarized as follows: 11 survived infection with Type III, and 2 showed evidence of increased resistance. Expressed in percentage, 73 per cent survived and a total of 86 per cent showed evidence of active immunity.

Group III. Rabbits Immunized with R Forms of Pneumococci.

Eleven rabbits belong to this group. 5 were immunized with an R strain derived from Type I pneumococcus (designated R_1); 5 with an R strain derived from Type II (designated R_2); 1 with an R strain derived from Type III (designated R_3). The sera of these rabbits contained no type-specific antibodies, but only antiprotein antibodies reactive with R strains in dilutions of 1:160 to 1:320. Of the 5 rabbits immunized with R_1, 3 survived, 1 lived 6 days with evidence of resistance, and 1, living 4 days, was classified as not being immune. Of the 5 immunized with R_2, 4 survived, and 1 lived 5 days with evidence of resistance. The 1 animal immunized with R_3 survived. The results with the rabbits immunized with non-type-specific, non-encapsulated R strains show that 8 survived, 2 showed evidence of increased resistance, and 1 was not considered as immune. Expressed in percentage, 72 per cent survived and a total of 90 per cent showed evidence of increased resistance.

Group IV. Rabbits Immunized with Solutions of Pneumococci.

Six rabbits belong to this group. The sera of these animals contained only antiprotein antibodies. The titre of R agglutinins obtained by immunization with purified nucleoprotein (Rabbits 24 and

25 in Table III) was 1:100. The antiprotein antibodies present in the sera of the rabbits immunized with desoxycholate solution of pneumococci (Rabbits 27, 28, 29, and 30) agglutinated R organisms in dilutions of 1:1260. No type-specific antibodies were demonstrable.

None of these animals showed evidence of increased resistance to the amount of culture with which they were infected. Although 1 lived $3\frac{1}{2}$ days, blood cultures showed countless organisms (∞) in each culture taken later than 18 hours after infection. Table III shows the course of the bacteremia in this group of animals, which is in striking contrast to the blood infection occurring in resistant animals (Tables I and II).

Analysis of Results.—The data presented in the foregoing experiments reveal the interesting fact that of 38 rabbits which had been previously immunized with pneumococci 25 survived the intravenous injection of living, virulent Type III organisms in amounts at least a thousandfold greater than the dose invariably fatal for normal controls. A second point of equal interest is the fact that this solid immunity against Type III infection may be established by preliminary treatment with cells of heterologous types and with the degraded R forms of pneumococci. In other words, this form of active immunity is effective in the complete absence of demonstrable type-specific antibodies in the serum of the treated rabbits, and appears to be unrelated to the variety of pneumococcus used for immunization. In attempting to analyze the mechanism underlying this form of effective but non-specific resistance recourse was had to the method previously described (2) by means of which the course of the infection may be followed by observing the intensity and duration of the bacteremia. The course of the bacteremia in 7 of the infected rabbits is given in Table I. The figures presented in the table are typical and representative of the results obtained in the group of animals surviving infection, regardless of the variety of pneumococcus used for immunization, and are equally characteristic of the normal control group. 3 of the animals whose protocols are given, had been previously immunized with a rabbit avirulent strain of Type III, 3 others had been similarly treated with R cells derived from heterologous types, and the remaining rabbit had been immunized with Pneumococcus

Type II. For purposes of comparison the course of the bacteremia during the fatal infection of 2 normal control rabbits is included in the same protocol. The rapidly fatal septicemia in the non-immune animals with progressively increasing and overwhelming numbers of organisms constantly in the blood is in striking contrast to the mild,

TABLE I.

Course of Bacteremia in Immunized Rabbits Surviving Infection with Rabbit Virulent Strain of Pneumococcus Type III.

(The results given in this protocol are representative of 25 rabbits which survived.)

Rabbit No.....	1	2	3	4	5	6	7	8	9
Pneumococcus used for immunization	Normal control	Normal control	Type III	Type III	Type III	Type II	R_1 strain	R_1 strain	R_2 strain
Time of culture	No. of colonies per unit of blood								
15 min.	∞	∞	49	660	164	261	3200	620	119
2 hrs.	107	∞	1	2	0	12	22	0	37
5 "	393	∞	0	0	3	5	6	1	20
12 "	562	∞	0	6	0	9	12	8	
24 "	∞	D	0	18	0	38	18	3	216
36 "	∞		0	0	4	72	6	10	12
48 "	D		0	0	23	4	5	176	32
72 "			0	0	14	0	5	14	4
4 days			0	0	30	29	3	342	35
5 "			S	S	7	14	15	66	5
6 "					4	6	1	8	6
7 "					7	2	0	6	0
8 "					2	3	6	4	0
9 "					4	0	0	2	0
10 "					1	0	0	0	0
11 "					0	0	0	0	0
					S	S	S	S	S

S indicates survival of animal.
D indicates death of animal.

fluctuating, but progressively decreasing bacteremia which characterizes the course of infection in the immunized rabbits. In many instances, the blood infection in the immune animals may persist for several days, the circulating organisms varying in number from time to time before ultimately disappearing. The non-fatal course of the bacteremia in the treated animals following infection with an S strain

of Type III, highly virulent for rabbits, parallels very closely the curve of the benign bacteremia (2) which occurs in normal rabbits infected with an S culture of Type III, avirulent for this species. The

TABLE II.

Course of Bacteremia in Immunized Rabbits Not Surviving Infection with Rabbit Virulent Strain of Pneumococcus Type III.

Rabbit No.	10	11	12	13	14	15	16	17	18	19	20	21	22
Pneumococcus used for immunization	Normal controls		Type III	Type III	Type III	Type III	Type III	Type III	Normal controls		R_2	R_1	R_1
Time of culture													
15 min.	∞	∞	29	168	∞	184	330	164	∞	∞	268	4000	564
2 hrs.	∞	3200	9	253	96	15	3	1	3000	∞	4	28	3
5 "	∞	∞	2	406	13	22	3	0	∞	∞	3	5	5
12 "	∞	∞	5	592	24	90	38	0	∞	∞	0	7	9
24 "	∞	∞	6	D	8	15	314	0	∞	D	34	2	2
36 "	D	D	1		4	D		0	D		103	83	5
48 "			251		12		328	23			316	41	4
72 "			133		3		19	14			56	9	24
4 days			316		8		42	30			38	D	33
5 "			93		12		35	7			D		123
6 "			116		216		21	4					D
7 "			D				1200	7					
8 "					72		86	2					
9 "					1500			4					
10 "					D		10	1					
11 "							4	0					
12 "								2					
13 "								1					
14 "							20	6					
15 "							32						
16 "							D						
17 "								3					
18 "													
19 "													
20 "								1					
21 "								D					

D indicates death of animal.

possible significance of the similarity in the course of the bacteremia in both instances and its relation to the mechanism of recovery will be discussed later.

Of the 38 rabbits immunized with pneumococci—25 of which completely recovered from virulent Type III infection—there were 13 animals which died. However, 9 of these may justly be considered as having acquired a considerable though ineffective degree of immunity as a result of the previous immunization. These animals lived 5 to 21 days following infection, whereas the controls all died within 24 to 36 hours. As previously stated, all the experimental animals suffered a massive infection receiving 1 cc. of the virulent Type III culture representing from 1000 to 10,000 minimal lethal doses. If the test had been made less severe by giving smaller infecting doses the number of surviving animals would, in all probability, have been greater.

Not only the duration of life but also the degree of the bacteremia evidenced the presence of resistance in these non-surviving animals. In Table II is given the course of the blood infection in 9 rabbits as estimated by blood cultures taken at frequent intervals. From the table it may be seen that there is an initial sharp reduction in the number of circulating organisms in the resistant animals as contrasted with the controls, and that, although death eventually ensued, the blood infection during life ran a moderately low grade and irregular course, not unlike that in the surviving rabbits. Even in 2 animals (Nos. 13 and 15), which died within 48 hours, and not tabulated as immune, the extent of the bacteremia is markedly less than in the controls. The results indicate that these partially resistant rabbits, although not possessing a solid immunity, were capable of checking the infection, either by inhibiting multiplication of the bacteria or by actually destroying them. Still further evidence that the rabbits, in which death was delayed, possessed some immunity is brought out by the fact that at autopsy, of the 9 examined, 7 suffered from purulent pericarditis and pleuritis, a condition not found in the normals, which died of an overwhelming septicemia. Localization of infection is generally considered as evidence of partial immunity and this has been especially emphasized by Stillman (6) in experimental production of lobar pneumonia. It seems highly probable that the local inflammatory processes were at least partially responsible for the fatal outcome.

In striking contrast to the effective resistance against Type III infection acquired by rabbits immunized with pneumococcus cells is

the absence of protection in other animals immunized with solutions of heterologous pneumococci. The course of the infection in this group shows that they possessed no resistance, at least against a dose as great as 1 cc. of virulent culture. 5 of the 6 rabbits died within 48 hours and 1 lived $3\frac{1}{2}$ days. The bacteremia in these animals was only transiently reduced or entirely unaffected (Table III). Furthermore, none possessed evidence of localization of infection on gross post-mortem examination. It is a striking fact that although these rabbits possessed circulating antiprotein antibodies (anti-P) similar to the rabbits immunized with whole organisms, no increased resistance was

TABLE III.

Course of Bacteremia in Rabbits Immunized with Solutions of Pneumococci and Injected with Rabbit Virulent Strain of Pneumococcus Type III.

Rabbit No.	23	24	25	26	27	28	29	30
Material for immunization	Normal control	Pneumococcus nucleoprotein		Normal control	Desoxycholate solution of pneumococcus (R_2)			
15 min.	∞	∞	∞	∞	∞	∞	∞	∞
2 hrs.	∞	∞	∞	∞	175	∞	115	∞
5 "	714	∞	∞	∞	426	∞	217	∞
12 "	∞	∞	∞	∞	∞	∞	382	∞
24 "	∞	D	∞	D	∞	D	∞	∞
36 "	D		D		∞		∞	D
48 "					∞		D	
72 "					∞			
96 "					D			

D indicates death of animal.

apparent. This is strongly suggestive that anti-P antibodies, in themselves, are not significant in this form of active immunity.

DISCUSSION.

The experiments reported in the present paper demonstrate that a considerable degree of increased resistance against virulent Type III pneumococci may be stimulated in rabbits by immunization with homologous or heterologous type-specific S pneumococci or with R forms derived from them. The protection was equally effective regardless of the type of pneumococcal cells used for immunization

(Table IV). The exclusion of a type-specific immune reaction by the use of animals previously treated with heterologous S and R strains, makes it necessary to investigate other factors which might afford an explanation of the active immunity. Singer and Adler (8), in dealing with this problem, concluded that the resistance of immunized rabbits to Type III was dependent upon changes (*Umstimmung*) in reticulo-endothelial cells with which was associated the ability to phagocyte the virulent organisms. Interesting as their experiments were, they were inconclusive in excluding the possible influence of sessile specific antibodies. However, when heterologous S and R

TABLE IV.

Summary of Results in Rabbits Immunized with Homologous or Heterologous Pneumococci and Subsequently Infected with Rabbit Virulent Strain of Type III.

No. of rabbits	Immunized with	Type III agglutinins	R agglutinins	No. survived	No. resistant not surviving	Total No. resistant	Per cent	No. not resistant	Per cent
12	Type III	3+ 9−	+	6	4	10	83	2	17
15	10 Type II 5 Type I	−	+	11	2	13	86	2	14
11	R	−	+	8	2	10	90	1	10
6	Pneumococcus solutions	−	+	0	0	0	0	6	100

pneumococci afford protection, as the present study indicates, type-specific immunological reactions are entirely eliminated. Wright (9) in a recent publication has reported the results of extensive studies on pneumococcus immunity. Employing Type I pneumococci he was able to demonstrate active immunity in rabbits although demonstrable agglutinins were not present in the sera of the immunized animals. However, under the conditions of his experiments, he did not obtain increased resistance to Type I by previous injection of heterologous organisms and concluded that the reaction was type-specific.

Although the experiments reported in this paper have been carried out under conditions which entirely exclude type-specific immunity,

nevertheless, the favorable results obtained under such conditions in no sense minimize the thoroughly established significance of type-specific antibodies in protection against pneumococcus infection. Their effectiveness in sensitizing virulent homologous pneumococci and thereby making phagocytosis possible, has been repeatedly observed.

In seeking for an explanation of the form of non-specific immunity against Type III infection on the basis of circulating antibodies it may be noted that the sera of all the immune animals possessed antiprotein (anti-P) antibodies. Antibodies of this character are reactive with the common pneumococcus nucleoprotein (precipitin) and with all R strains (agglutinin). They are not, however, reactive with encapsulated type-specific organisms nor do they confer passive protection on mice against virulent pneumococci. Consequently it is highly improbable that they are responsible for the disposition of pathogenic Type III organisms. More direct evidence of the ineffectiveness of these antibodies in resistance is brought out by the fact that immunization with pneumococcus solutions (derived from heterologous organisms) fails to afford protection although the sera of rabbits so treated possess anti-P antibodies in high titre. The one factor which all the resistant animals had in common was immunization with formed pneumococcal cells. The nature of the material used as antigen rather than the demonstrable antibody response, therefore, seems to be the significant feature in stimulating this form of active immunity.

Since type-specific antibodies have been excluded, and since the presence of species antibodies (anti-P) does not furnish an adequate explanation for the resistance to Type III, it is necessary to seek further for an understanding of this form of immunity. In a previous paper (2) it was shown that rabbits possess a considerable degree of natural resistance to Pneumococcus Type III, although the strains used for injection were S forms and highly pathogenic for mice. It was also shown that the blood infection resulting from the injection of these *rabbit avirulent* organisms into *normal animals* is characterized by a prolonged course during which the number of circulating bacteria vary from time to time but eventually disappear. In the present paper it is shown that the bacteremia occurring in an *immune rabbit* injected with a *rabbit virulent* strain runs a strikingly similar course.

Consequently it seems possible that the explanation of acquired resistance, in this instance, is due not to antibodies which have been elicited, but to an increased effectiveness of the mechanism of natural resistance. Wright (9) in his recent publication has offered a similar explanation for the active immunity which he obtained and he considered the difference between normal and immune rabbits to be quantitative and not qualitative. However, the emphasis which he places upon specificity makes it necessary to assume that normal rabbits possess type-specific protective susbtances, a conception which Sia (10) previously suggested and more recently (11) has further emphasized. The experiments reported in this paper reveal the fact that enhanced resistance to Type III pneumococci may be stimulated in rabbits by previous injections of any *R* or *S* pneumococcus cells, and, according to the explanation advanced, is due to an exaltation of the same factors which endow normal rabbits with natural resistance to Type III infection. Further experiments tending to substantiate this view will be subsequently reported. If this hypothesis proves correct, these experiments also tend to show that, whereas intact cells stimulate the processes of natural resistance, the same material in solution is ineffective, although a similar antibody response (anti-P) is elicited in both instances.

SUMMARY.

1. Immunization of rabbits with Type III pneumococci is effective in producing active immunity against infection with a virulent strain of the homologous organism.

2. Immunization of rabbits with Type I or II pneumococci, and with R forms derived from any of the fixed types, is equally effective in producing active immunity against Type III infection.

3. Immunization of rabbits with nucleoprotein or with desoxycholate solutions of heterologous pneumococci, under the experimental conditions described, appears to be ineffective in producing active immunity against Type III infection.

CONCLUSIONS.

Increased resistance against virulent Type III pneumococci may be stimulated in rabbits by repeated injections of heat-killed cultures

of homologous or heterologous pneumococci. This form of active immunity, effective in the absence of demonstrable type-specific antibodies and unrelated to the variety of the pneumococcus used for immunization, is considered dependent upon an exaltation of the same factors which afford normal rabbits natural resistance to Type III pneumococcus.

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