REACTIONS OF RABBITS TO INTRACUTANEOUS INJECTIONS OF PNEUMOCOCCI AND THEIR PRODUCTS

II. Resistance to Infection

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The results reported in the preceding communication (1) disclose the fact that the antibody responses to the injection of heat-killed pneumococci differ qualitatively, and that the nature of the response is related to the route of administration of the organisms. Pneumococci of Type I, which are invariably effective in stimulating the formation of type-specific antibodies when they are introduced intravenously, were ineffective in producing this type of antibody response in 88 per cent of the rabbits when the injections were made intracutaneously. Under the latter conditions, the antibody response was solely or predominantly species-specific and was no different from the response of rabbits to the intracutaneous injection of S cells of different types, or, indeed, of degraded, non-type-specific R organisms.

The question naturally suggests itself whether rabbits which have received repeated intracutaneous injections of heat-killed pneumococci, and therefore have in their sera no type-specific antibodies, are, nevertheless, actively resistant to infection, and if so, whether this form of acquired resistance is likewise effective against infection by heterologous types of Pneumococcus. The question also arises whether the sera of animals immunized by intracutaneous injections will confer passive protection upon white mice against infection by homologous types of Pneumococcus. A preliminary report (2) has already been made of the experiments here presented which were undertaken to solve these problems.

EXPERIMENTAL

Rabbits were immunized by repeated intracutaneous injections. The inoculations were made once a week and consisted of 0.2 cc. of a suspension of heat-killed

bacteria, or the equivalent of 2.0 cc. of broth culture. The degree of resistance to infection was determined 10 to 21 days after the last intracutaneous inoculation by injecting intravenously varying quantities of live virulent cultures of Type I or Type III pneumococci. The Type I strain killed normal rabbits in amounts of 10^{-7} cc. of broth culture, while the Type III strain was fatal in an amount of 10^{-3} cc. to 10^{-4} cc. Samples of blood were obtained from the rabbits immediately preceding infection and the sera were used to determine the titre of type-specific agglutinins and also the power to protect white mice against infection by pneumococci of homologous types.

Resistance to Infection Following Intracutaneous Immunization with Heat-Killed "S" Cells of Pneumococcus

I. Immunization with Type I (S) Pneumococcus

Forty rabbits were immunized intracutaneously to Type I Pneumococcus and tested later for their resistance to infection. In the earlier experiments the resistance to infection by the homologous type of Pneumococcus was determined, while in the later experiments the degree of active immunity to infection by some type of Pneumococcus other than that used for immunization was tested.

(*a*) *Active Resistance to Infection by Pneumococcus Type I (Homologous)*. Resistance to infection by the homologous type of Pneumococcus was first determined in a group of three rabbits (Table I).

Each had received into the skin 12 injections of suspensions of heat-killed pneumococci, the total number of bacteria injected being equivalent to those contained in 27 cc. of broth culture. Twelve days after the last inoculation all the animals were given intravenously 0.1 cc. of living virulent Type I culture, and all survived. Control, normal rabbits died within 48 hours after receiving 0.0,000,001 cc. of the same culture. The serum of one of the 3 immunized rabbits possessed an agglutinin titre for the type-specific organism of 1:1; sera from the other two rabbits had no agglutinating power. A second group of 9 rabbits received 8 injections of heat-killed pneumococci equivalent to those contained in 16 cc. of broth culture. Subsequently they survived the intravenous injection of live cultures in quantities as large as 0.2 cc., whereas normal rabbits succumbed following injections of 0.0,000,001 cc. In only 3 of the rabbits were type-specific agglutinins demonstrable.

These observations demonstrate that intracutaneous immunization with heat-killed Type I pneumococci is followed by resistance to the intravenous injection of virulent cultures of the same type.

(*b*) *Active Resistance to Infection by Type III Pneumococci (Heterol-*

ogous). The foregoing experiment shows that rabbits may acquire an effective resistance to infection by the same organism used in immunization, even when demonstrable circulating type-specific antibodies are absent. Consequently it was interesting to determine whether this form of resistance is also effective against infection with pneumococci of heterologous types. Accordingly, in all the following experiments resistance was determined to infection by Pneumococcus Type III. In Table I are given results of these experiments in 31 rabbits. It is evident that following intracutaneous administration of suspensions of heat-killed Type I pneumococci, rabbits acquire a marked degree of resistance to infection by virulent Type III organisms. In a general way, the resistance increases with the number of injections. However, in the last experiment given in Table I the rabbits received only one injection and yet they were resistant to 100 lethal doses of Pneumococcus Type III.

(*c*) *Passive Immunity.* It is seen, then, that rabbits may be actively immunized by intracutaneous injections, not only against pneumococci of the type used in immunization but also against pneumcococci of other types, and this in the absence of circulating type-specific antibodies. It was interesting to determine whether the sera of these rabbits protected white mice against infection. Protection tests were conducted in the usual manner by the simultaneous intraperitoneal injection of 0.2 cc. of serum and varying dilutions of Type I Pneumococcus culture. The sera of 53 animals were tested (Tables I and III); the sera of 42 of them showed no measurable protective properties for mice, while the sera of the remaining 11 exhibited protective power against only very small infecting doses of Pneumococcus Type I culture. Of the 11 sera which were protective, 6 contained no type-specific agglutinins while 5 showed type-specific agglutinin titres of 1:1–1:20. On the other hand, of the 42 sera with no protective capacity only one showed type-specific agglutinins, and in this case the agglutination titre was 1:3. It is evident, therefore, that in most instances in which the sera possessed protective power this was associated with the presence of type-specific agglutinins. On the other hand, in most of the animals the sera possessed no protective power for mice and no type-specific agglutinins. It is possible, however, as the work of Tillett (3) suggests, that these sera might have protective power for rabbits,

TABLE I

Resistance of Rabbits to Infection Following Intracutaneous Immunization with Heat-Killed Pneumococcus, Type I

Number of rabbits	Number of injections	Total amount injected	Immunity to Type I (homologous)			Immunity to Type III (heterologous)			Protective capacity for mice		Anti-S titre
			cc.	Survived	Died	cc.	Survived	Died	Number of sera	Degree of protection	
		cc.									
									2	none	negative
3	12	27	0.1	3	—	—	—	—	1	10^{-5}	1–1:1
									1	10^{-2}	1:20
9*	8	16	0.2	3	—	1.0	1	1	1	10^{-3}	1:1
									2	10^{-4}	1:5
			0.1	3	—	0.8	2	1	2	10^{-5}	negative
									1	10^{-6}	negative
			0.01	3	—	0.5	3	—	2	none	negative
5	7	14	—	—	—	—	—	—	5	none	negative
4	14	29	—	—	—	1.0	2	—	4	none	negative
						0.5	2	—			
5	10	20	—	—	—	1.0	—	2			
						0.8	—	2	5	none	negative
						0.5	1	—			
2	5	10	—	—	—	1.0	1	—			
						0.5	1	—	2	none	negative
2	10	20	—	—	—	1.0	1	—	1	10^{-6}	negative
						0.5	1	—	1	none	negative
9	1	2	—	—	—	0.5	—	1			
						0.3	—	2	9	none	negative
						0.1	—	2			
						0.01	2	—			
						0.001	2	—			

M. L. D. of Type I culture for normal rabbits, 10^{-7} cc.; for mice 10^{-7} cc.

M. L. D. of Type III culture for normal rabbits, 10^{-4} cc.; for mice 10^{-7} cc.

* In this instance, all nine rabbits were tested first for resistance to Type I infection; and later to Type III.

but this question has not been studied. He observed that, following intravenous immunization with R pneumococci, rabbits not only acquired an active resistance but that the sera of these animals, passively transferred to other rabbits, protected them against infection with pneumococci of every type. These sera, however, afforded no protection to mice against pneumococcal infection.

II. Immunization with Type III (S) Pneumococcus

Thirty-eight rabbits were immunized by intracutaneous injections of heat-killed Type III pneumococci (Table II). These animals were subsequently tested for active immunity to virulent cultures of Type I or Type III Pneumococcus.

(*a*) *Active Immunity to Infection by Type III (Homologous) Pneumococcus.* Resistance to infection by pneumococci of the homologous type was tested in 9 rabbits.

They were immunized by 8 injections into the skin of heat-killed pneumococci Type III, the total number of bacteria corresponding to those contained in 16 cc. of broth culture. All the animals were subsequently infected by intravenous injections of varying amounts of culture in quantities as large as 1.0 cc. The culture employed killed normal rabbits in doses of 0.0001 cc. One of the animals died and 2 survived after receiving 0.5 cc. of culture, 2 died and 1 survived after 0.8 cc., and 1 died and 2 survived after 1.0 cc. of culture. In the 4 animals which succumbed, death was delayed from the seventh to the tenth day after the infection, and in each instance, the post-mortem examination revealed a massive pericarditis and pleurisy. The delayed death and localization of the infection may be considered to be an expression of active immunity.

It is obvious that these animals showed a considerable degree of active immunity to infection with homologous organisms.

(*b*) *Active Immunity to Infection by Type I Pneumococcus (Heterologous).* Twenty-eight rabbits which had been immunized by the intracutaneous injection of heat-killed pneumococci (Type III) were later tested for their immunity against infection with heterologous (Type I) pneumococci (Table II). The culture employed in the tests was of such a virulence that 0.0,000,001 cc. injected intravenously regularly killed normal rabbits. The immunized animals received doses of from 0.001 to 0.2 cc. Of the 28 immunized animals receiving these large doses, 22 recovered and only 6 died. It is evident, therefore, that the animals immunized by intracutaneous injections had

acquired a marked resistance against pneumoccci of a heterologous type.

(*c*) *Passive Immunity.* It is well known that only in rare instances is the serum of a rabbit immunized intravenously to Type III Pneu-

TABLE II

Resistance of Rabbits to Infection Following Intracutaneous Immunization with Heat-Killed Pneumococcus, Type III

Number of rabbits	Number of injections	Total amount injected	Immunity to Type III (homologous)			Immunity to Type I (heterologous)			Protective capacity for mice		Anti-S titre
			cc.	Survived	Died	cc.	Survived	Died	Number of sera	Degree of protection	
		cc.									
9	8	16	1.0	2	1						
			0.8	1	2				9	none	negative
			0.5	2	1						
3	12	27	—	—	—	0.01	3	—	3	none	negative
5	7	14	—	—	—	—	—	—	5	none	negative
4	14	29	—	—	—	0.1	1	1			
						0.01	2	—	4	none	negative
5	10	20	—	—	—	0.2	—	1			
						0.1	1	—	5	none	negative
						0.01	1	1			
6	7	14	—	—	—	0.1	1	1			
						0.01	2	—	—	—	—
						0.001	2	—			
5	7	14	—	—	—	0.1	2	—			
						0.01	1	1	5	none	negative
						0.001	1	—			

mococcus protective for white mice against infection with homologous organisms. In the experiments here recorded (Table II) the sera of 37 rabbits which were immunized intracutaneously with suspensions of heat-killed pneumococci Type III were tested for their protective power in white mice, and in no instance did these sera confer any

measurable protection. Moreover, in none of the sera were any type-specific antibodies demonstrable.

According to the recent work of Sia (4) normal pig serum, though lacking type-specific agglutinins, protects mice against infection by Pneumococcus of any type. The special technique which he employed was used in retesting six of the sera mentioned above. Three of these sera were from the animals immunized by the injection of Type III pneumococci and three from the rabbits immunized with Type I pneumococci. The serum was injected intraperitoneally into the mice 4 hours preceding the inoculation of the live culture. No protective power could be demonstrated in any of these sera by this method.

The Rate of Development and Duration of Active Immunity Following Intracutaneous Injections

Having determined that rabbits, following repeated intracutaneous immunization, acquire a marked resistance to infection by Pneumococcus of any type, observations were next made to determine how rapidly this form of active immunity develops and how long it persists.

Fourteen rabbits were immunized, employing a varying number of intracutaneous injections and different amounts of heat-killed bacteria (Table III (a)). Each of two animals received from 1 to 7 injections of the bacteria, corresponding to 2 cc. to 14 cc. of Type I pneumococcal culture. Three weeks after the last injection, the immunity to infection was measured against Type III pneumococci.

The results show that even after one intracutaneous injection of pneumococci, in an amount corresponding to 2.0 cc. of culture, rabbits may show a distinct resistance to infection with heterologous pneumococci. Of the 14 rabbits, the sera of only 2 showed any anti-S agglutinating power. In one rabbit, which had received 4 injections, the agglutination titre of the serum was 1:3, and in the other, which had received 7 injections, the titre was only 1:5. The protective action of these sera for mice was very low and irregular, only 3 sera affording minor protection against homologous infection. Curiously enough, the 2 sera possessing specific agglutinins afforded no protection to white mice.

Six rabbits of a second group (Table III (b)) were each immunized

TABLE III

Rate of Development and Duration of Immunity in Rabbits Immunized with Heat-Killed Pneumococcus Type I by the Intracutaneous Route

(a) Rate of Development

Number of rabbits	Number of injections	Total amount injected	Immunity to Type III (heterologous)			Protective capacity for mice		Anti-S
			cc.	Survived	Died	Number of sera	Degree of protection	
		cc.						
2	1	2	0.01	1	—			
			0.01	1	—	2	none	negative
2	2	4	0.1	1	—			
			0.3	1	—	2	none	negative
2	3	6	0.3	1	—			
			0.5	1	—	2	none	negative
2	4	8	0.5	1	—	1	none	negative
			0.5	1	—	1	none	1:3
2	5	10	0.5	1	—	1	none	negative
			0.8	1	—	1	10^{-5}	negative
2	6	12	0.8	1	—	1	10^{-6}	negative
			1.0	1	—	1	none	negative
2	7	14	0.5	—	1	1	none	negative
			1.0	1	—	1	none	1:5

(b) Duration of Immunity

Animals Immunized with Pneumococcus, Type III

Number of rabbits	Number of injections	Total amount injected	Immunity to Type I (heterologous) after 6 months			Protective capacity for mice	Anti-S
			cc.	Survived	Died		
		cc.					
1	8	16	10^{-1}	—	1	All sera negative both 10 days and 6 months after final injection	
1	8	16	10^{-2}	—	1		
1	8	16	10^{-3}	—	1		
1	8	16	10^{-4}	1	—		
1	8	16	10^{-5}	1	—		
1	8	16	10^{-6}	1	—		

by intracutaneous injections of a suspension of Type III organisms corresponding to a total amount of 16 cc. of culture. Six months after the final injection the animals were infected with Type I Pneumococcus culture in amounts varying from one-tenth to one-millionth cc. As shown in the table the animals were resistant to infection with one-ten-thousandth cc. It appears, therefore, that resistance to heterologous infection still persists after six months although at this time it is considerably diminished.

Resistance to Infection Following Intracuaneous Immunization with Suspensions of Heat-Killed R Cells of Pneumococcus

The R strain used for immunization was originally derived from a Type II Pneumococcus. Rabbits were immunized by intracutaneous injections of suspensions of heat-killed pneumococci in a manner similar to immunization with S cells. The animals received amounts of suspension equivalent to those usually employed in intravenous immunization. Resistance to infection was measured by injecting intravenously different quantities of a virulent culture of Type I pneumococci. The results given in Table IV show that 4 rabbits immunized to R pneumococci survived following the injection of 0.01 cc. or 0.1 cc. of a Type I Pneumococcus culture which killed normal rabbits in a dilution of 0.0,000,001 cc. The sera of the rabbits immunized with R organisms derived from Type II pneumococci afforded white mice no protection against infection by Type II pneumococci. The sera also did not agglutinate Type II pneumococci.

Resistance to Infection Following Intracutaneous Immunization with Soluble Derivatives of Pneumococcus

It has previously been shown that following intracutaneous immunization with S or R pneumococci, rabbits acquire an active immunity to infection by homologous and heterologous types of pneumococci. It was also shown in a previous paper (1) that the antibody response in the majority of rabbits is the same (species-specific) whether intracutaneous immunization is brought about by the injection of formed cells or by the injection of soluble derivatives of Pneumococcus. The present observations were made to determine whether, following intracutaneous injections of solutions containing soluble derivatives of

Pneumococcus, rabbits become actively immune to infection with living cultures.

The animals received (a) a solution of "nucleoprotein," and (b) the supernatant fluid after acid precipitation of the "nucleoprotein" from a solution of pneumococci resulting from repeated freezing and thawing. These solutions were prepared from a culture of Type II Pneumococcus in the same manner as described in the previous paper (1).

The rabbits receiving "nucleoprotein" were given repeated injections, the total amount of protein administered to each animal, as estimated by nitrogen deter-

TABLE IV

Resistance of Rabbits to Infection Following Intracutaneous Immunization with R Forms and with Soluble Protein Derivatives of the Cell

Antigen administered	Number of rabbits	Quantity of antigen	Immunity to Type I (heterologous)			Protection conferred by sera on mice (homologous infection)		
			cc.	Survived	Died	Number of sera	Degree of protection	Anti-S titre
R Pneumococcus Derived from Type II "S"	4	20 cc.	10^{-1}	2				
			10^{-2}	2		4	none	negative
Nucleoprotein Derived from Type II "S"	4	70 mgm.	10^{-3}	—	1			
			10^{-4}	—	1			
			10^{-5}	—	1	3	none	negative
			10^{-6}	1	—	1	10^{-6}	negative
Supernatant after removal of nucleoprotein	4	30 mgm.	10^{-3}	—	1			
			10^{-4}	—	1			
			10^{-5}	—	—	3	none	negative
			10^{-6}	1	—	1	10^{-6}	negative

mination, being 70 mg. Three weeks following the last immunizing injection, they were given intravenously varying quantities of a virulent Type I Pneumococcus culture. The results, as shown in Table IV, indicate that the immunity induced in these animals was extremely slight; indeed it may be doubted whether any increased resistance was demonstrated. In the sera of none of these animals were type-specific antibodies demonstrable and only one of the sera possessed any protective power for mice, in this case of minimal degree.

The rabbits receiving the second material were given repeated injections, the total amount of solution containing 30 mgm. of protein as estimated on the basis of nitrogen determinations. Active immunity was determined by observing the

results of the subsequent injection of different amounts of Type I Pneumococcus culture. The data are given in Table IV and it is seen that little immunity follows the intracutaneous injection of this material. The sera of none of the rabbits possessed type-specific antibodies and the serum of only one of them protected mice against a minimal infective dose. The sera of these rabbits, however, did contain species-specific antibodies, as was shown by their power to precipitate the "nucleo-protein" and to agglutinate R cells.

The results show, then, that repeated injections of soluble derivatives of Pneumococcus into the skin do not render rabbits resistant to infection. Tillett (5) has already pointed out that rabbits do not acquire an active immunity following intravenous immunization with solutions of Pneumococcus.

TABLE V

Protective Property of Sera of Rabbits Immunized Intravenously after Previous Intracutaneous Immunization

Number of rabbits	Previous immunization	Later immunization	Protection titre for mice	
			Number of sera	Degree of protection
				cc.
6	Intracutaneous Type I	Intravenous Type I	3	0.2
			3	0.1
6	Intracutaneous Type III	Intravenous Type I	3	0.2
			2	0.1
			1	0.01

Protective Properties of Sera of Rabbits Immunized Intravenously Following a Previous Intracutaneous Immunization

It was shown in the preceding communication (1) that previous intracutaneous immunization does not render rabbits incapable of forming type-specific antibodies when they are subsequently given intravenous injections. It was interesting to determine the protection titre of the sera of rabbits immunized first by intracutaneous inoculations and later by intravenous injections. The data presented in Table V show that 12 rabbits were originally immunized by intracutaneous injections of suspensions of heat-killed pneumococci. Six were immunized to Type I and six to Type III pneumococci. At the end of the immunization, none of the sera conferred protection upon

white mice to infection by pneumococci of the respective types, and none contained type-specific antibodies. After a subsequent immunization by intravenous injections of Type I pneumococci the sera of all 12 rabbits not only showed a high agglutination titre (about 1:160) but all conferred a high grade of passive protection upon mice against infection by Pneumococcus Type I.

DISCUSSION

The results of the preceding communication (1) disclose the fact that immunization by the intracutaneous injection of suspensions of heat-killed pneumococci, whether S or R, incites the formation of species-specific antibodies in all rabbits. In the animals immunized with Type III pneumococci no type-specific antibodies were found, and in the rabbits immunized with Type I pneumococci antibodies appeared in only 12 per cent of the rabbits studied. The present study reveals that following intracutaneous immunization rabbits acquire a marked degree of resistance to intravenous infection by pneumococci, and that this is true whether the pneumococci injected be of the same type as those employed in immunization or of a different type. This active immunity is effective even in the absence of type-specific agglutinins.

In general, the sera of rabbits immunized by intracutaneous injections fail to protect white mice against infection by Pneumococcus. However, when pneumococci of Type I are employed in the immunization, about 20 per cent of the sera studied conferred on white mice some degree of protection against infection.

Tillett was the first to point out (5) that the intravenous immunization with R or S cells of any type of Pneumococcus renders rabbits actively immune to infection with Type III Pneumococcus. In a subsequent communication Tillett showed (3) that although the sera of rabbits intravenously immunized to R or S pneumococci failed to protect white mice against infection by Pneumococcus, nevertheless, these sera did confer protection upon normal rabbits. Later Goodner (6) reported that rabbits recovering from an intracutaneous infection with live virulent cultures of Type I Pneumococcus were subsequently immune to infection with pneumococci of the same type. More recently Bull and McKee (7) found that following immunization of

rabbits by subcutaneous and intravenous injections of pneumococci of Type II or Type III and by similar injections of a strain of pneumococci of Group IV the animals were actively immune, and that this immunity was effective against intransal or intravenous injections of pneumococci of Type I. Species-specific antibodies were present in the sera of all the animals. Type-specific agglutinins were present in the sera of the rabbits immunized against Pneumococcus of Type II and in the serum of the animals immunized against a Pneumococcus belonging in Group IV. In previous papers (8, 9) Bull and McKee had demonstrated that rabbits in which cultures of Type I pneumococci had been instilled into the nose were subsequently actively immune to infection with the homologous organism, and this in the absence of demonstrable type-specific antibodies.

On the other hand, the intracutaneous injections of soluble derivatives of pneumococci in rabbits is not followed by an active resistance to infection with these organisms and the sera of these animals fail to protect white mice against infection.

The observations made in this study indicate, then, that an active and broad resistance to infection by Pneumococcus may be present in the absence of circulating type-specific antibodies. Active immunity to pneumococcal infection is usually considered to be associated with the development of demonstrable antibodies which in one way or another react specifically with the bacteria themselves. The sera of animals immunized in the usual manner by repeated intravenous injections usually afford passive protection against infection by corresponding types of Pneumococcus to animals of various species and the passive protection is considered to depend upon the transfer of type-specific antibodies. In rabbits immunized by intracutaneous injections, however, the active resistance to infection which develops has been found to be unrelated to type-specificity as is evidenced both by the absence of type-specific antibodies in the serum and by the fact that the resistance to infection which these animals exhibit is effective, not only against pneumococci of the homologous type, but also against infection with pneumococci of heterologous type. Moreover, the sera of these animals do not, as a rule, confer passive protection upon white mice against infection even by homologous types of pneumococci. It is possible that in the one case the resistance is dependent upon the development of a new property, an acquired *active immunity*, and that

in the other case the resistance depends upon an exaltation of a naturally occurring property, an increase in natural *resistance to infection*, but this cannot be determined from the data at hand.

SUMMARY AND CONCLUSIONS

1. Injection of suspensions of heat-killed pneumococci into the skin of rabbits is followed by an active immunity which is effective against intravenous infection by homologous and heterologous types of Pneumococcus.

2. This form of active immunity may be induced by the injection of S or R strains of Pneumococcus.

3. Intracutaneous immunization with soluble derivatives of Pneumococcus does not induce active immunity to infection.

4. The sera of seventy-nine per cent of the rabbits immunized to Type I Pneumococcus by intracutaneous injections afforded no protection to mice against infection with pneumococci.

5. None of the sera of rabbits intracutaneously immunized to the type-specific Type III (S) pneumococci, to R cells, or to soluble derivatives of Pneumococcus protected white mice against infection.

6. The sera of rabbits immunized first intracutaneously and subsequently intravenously possess a high titre of protective antibodies.

7. It may be concluded that when type-specific pneumococci are injected into the skin they lose the property of stimulating an active immunity of a specific type and of stimulating the production of type-specific antibodies, but they act just as do the degraded or R forms, causing the animals to become resistant to infection with pneumococci of all types without the development of any type-specific antibodies in the serum.

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