THE DEVELOPMENT OF AGGLUTININS AND PROTECTIVE ANTIBODIES IN RABBITS FOLLOWING INHALATION OF PNEUMOCOCCI.

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(Received for publication, February 21, 1927.)

In a preceding paper (1) it has been shown that mice may acquire a high degree of active immunity following repeated inhalations of living pneumococci. However, mice are not suitable for tracing serologically the immunity developed during a series of exposures. Since rabbits may be easily and repeatedly bled, they were chosen for this work.

It has already been shown that rabbits are susceptible to infection by inhalation of Type I pneumococci (2), and that an occasional rabbit may recover from pneumococcus septicemia. In the present paper are reported the development of (1) agglutinins and (2) protective antibodies in the blood serum of rabbits following repeated inhalations of virulent Type I pneumococci. The duration of active immunity and the length of time that agglutinins and protective antibodies persist in the serum will be dealt with in a subsequent paper.

Method.

Rabbits were placed in a large spray chamber similar to that already described (3) and exposed to a spray of virulent Type I pneumococci. 50 cc. of an 8 hour broth culture were used for each spraying. The animals were exposed at 10 day intervals. Before each spraying, a sample of blood was obtained from the ear vein of each animal.

The presence of agglutinins was determined by a modified thread reaction. To 1 cc. of rabbit serum diluted in normal salt solution was added 0.2 cc. of an actively growing broth culture of Pneumococcus Type I. The tubes were incubated for 2 hours in the water bath at 37°C., placed in the ice box overnight, and the reactions read the next morning. Agglutinins were recorded as present in the serum only when the reactions were positive in a dilution of at least 1:10.

The presence of protective antibodies in the blood of the sprayed rabbits was determined by the ability of 0.2 cc. of serum to protect white mice against intra-

peritoneal injection of 0.001 cc. of pneumococcus culture, of which 0.000,001 cc. killed a normal mouse within 48 hours. The rabbit serum and culture were administered simultaneously.

TABLE I.

Relation of Development of Agglutinins to the Number of Exposures.

No. of exposures	1	2	3	4	5	6	7	8	9	10
" " rabbits	135	108	87	63	48	36	34	31	25	23
" " " showing agglutinins.	4	13	12	14	12	9	9	8	6	6
Per cent of rabbits showing agglutinins	2	12	13	22	25	25	26	25	24	26

TABLE II.

First Appearance and Titre of Agglutinins.

Following exposure	1	2	3	4	5	6	7	8	9	10
Rabbit 1	—	1:10								
2	—	1:10								
3	1:50	1:50								
4	1:50	1:100								
5	—	—	—	1:20						
6	—	—		1:20						
7	1:20	1:20	1:20	1:20						
8	—	—	1:50	1:50						
9	—	1:20	1:20	1:20	1:20					
10	1:50	1:100	1:100	1:100	1:100					
11	—	1:10	1:20	1:20	1:20					
12	—	—	1:10	1:10	1:10	1:10				
13	—	—	—	—	—	—	1:10			
14	—	1:10	1:40	1:40	1:100	1:100	1:100	1:100		
15	—	—	1:10	1:20	1:100	1:100	1:100	1:100		
16	—	1:40	1:40	1:50	1:50	1:50	1:100	1:100	1:100	1:100
17	—	1:10	1:50	1:50	1:50	1:50	1:50	1:50	1:50	1:50
18	—	—	—	—	1:100	1:200	1:200	1:200	1:200	1:200
19	—	1:20	1:40	1:40	1:40	1:40	1:40	1:40	1:40	1:50
20	—	—	—	—	1:10	1:10	1:20	1:40	1:40	1:40
21	—	1:20	1:40	1:40	1:50	1:50	1:50	1:50	1:50	1:50

Development of Agglutinins.

Out of 231 rabbits sprayed from 1 to 10 times, 80 or 34 per cent died from pneumococcus septicemia. The greatest number of deaths occurred after the first exposure. The sera for the serological reactions

which form the basis of this paper, were obtained from rabbits which had not only the greatest natural resistance, but also a certain degree of immunity acquired as the result of repeated exposures to live pneumococci.

In Table I are shown the number of rabbits which were sprayed from 1 to 10 times, the number which died of pneumococcus septicemia, and the number whose serum showed agglutinins in dilutions of at least 1:10. From Table I it is seen that only 4 or 2 per cent of the 135 rabbits which survived the first exposure developed demonstrable agglutinins. The percentage of rabbits showing agglutinins rose after the 2nd spray to 12 per cent, increasing to 25 per cent on and after the 5th spray. In fact 17 rabbits, after having been sprayed 10 times, failed to develop demonstrable agglutinins.

In all, 21 rabbits developed agglutinins. In Table II are shown the exposure after which agglutinins first appeared and the titre of agglutinins following each subsequent spraying. Although 4 rabbits showed agglutinins after the first exposure, a second exposure was necessary to incite the formation of these antibodies in 9 others. 1 rabbit first showed agglutinins only after 7 exposures. Great variations are also seen in the antibody titre of the different animals. As a rule agglutinins were first demonstrable only in the higher concentrations of serum (1:10 or 1:20) but in one instance the reaction first appeared following the 5th spray and was then present in a dilution of 1:100. Following the initial appearance of agglutinins there is a tendency for the titre to rise after the next spray and then to remain stationary. Of 8 rabbits which were sprayed 8 times, only 3 showed an agglutinin titre of 1:100; the serum of 3 others showed a titre as high as 1:50, while in one exceptional instance the serum suddenly showed agglutinins after the 5th spray in a dilution of 1:100 increasing after the 6th spray to 1:200.

Protective Antibodies.

In order to determine whether the serum of normal rabbits contains any natural antibodies against Type I pneumococcus, 1 cc. of serum from each of 147 normal rabbits was injected intraperitoneally into as many mice. The next day these mice were injected intraperitoneally with 0.000,001 cc. of virulent culture of Type I pneumococcus.

136 or 92 per cent of these mice died. In order to determine whether the 11 surviving mice recovered by virtue of a natural antibody present in certain normal rabbit sera, or by reason of a non-specific reaction induced by foreign protein, plain broth and normal horse serum were tested. 25 mice received in like manner injections of 1 cc. of plain broth, and another 25 were injected with 1 cc. of normal horse serum. 24 hours later all 50 mice were inoculated intraperitoneally with 0.000,001 cc. of virulent Type I pneumococcus. Of the mice receiving a preliminary injection of broth 24 or 96 per cent died, and of those injected with horse serum 17 or 68 per cent succumbed to subsequent infection. From the results of this experiment it would seem that the mere prelim'nary injection of a foreign serum of either the horse or rabbit, in certain instances conferred protection in mice against an

TABLE III.

Number of Rabbits Exposed and Number Whose Serum Protected Mice against 0.001 Cc. of Pneumococcus Type I.

No. of exposures	1	2	3	4	5	6	7	8	9	10
" " rabbits	135	108	87	63	48	36	34	31	25	23
" " " whose serum protected	5	10	14	25	22	22	21	23	19	19
Per cent of rabbits whose serum protected	3	9	16	39	45	61	61	74	76	82

otherwise fatal inoculation of Type I pneumococcus. From this it is evident that the 8 per cent of normal rabbit sera which afforded protection did so not because of the presence of natural antibodies but because of the non-specific protective reaction induced by the foreign serum.

In Table III are shown the number of rabbits which were sprayed from 1 to 10 times with Type I pneumococci and the number thus exposed whose serum subsequently protected mice against intraperitoneal injection of 0.0001 cc. of a virulent culture of the homologous organism. From this table it is seen that protective antibodies were demonstrable in the sera of 5 or 3 per cent of the rabbits after the 1st spray. Following each successive spraying the number of rabbits showing protection steadily increased, until after the 10th spraying the

serum of 82 per cent of the animals conferred passive protection on mice against at least 1000 lethal doses of virulent culture. A total of 49 rabbits developed protective antibodies. The spray following which the rabbit sera first protected mice is shown in Table IV.

From Table IV it is seen that with each successive spray the number of rabbits in whose sera protective antibodies were demonstrable progressively increased. Although the greatest number of rabbits showed protective antibodies in their sera after the 6th spray, other rabbits did not develop these antibodies until after the 10th exposure.

Correlation of Agglutinins and Protective Antibodies.

It is difficult to compare the relative titre of the sera at any one time because of the difference in the standards used. Whereas agglutinins were recorded as positive if present in serum concentrations of 1:10,

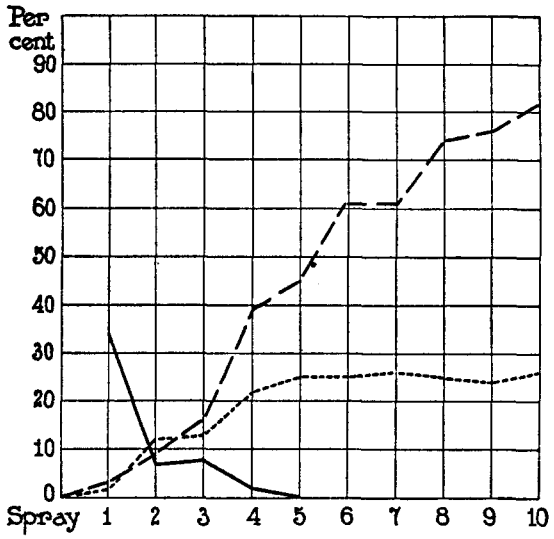
TABLE IV.

First Appearance of Protective Antibodies.

No. of spray	1	2	3	4	5	6	7	8	9	10
" " rabbits first showing protective antibodies	5	7	7	12	7	5	1	4	—	2

protective antibodies were noted only if they were present in concentrations sufficient to protect mice against a 0.0001 cc. of virulent pneumococcus culture. If smaller doses of Pneumococcus had been used, the presence of protective antibodies would undoubtedly have been detected earlier and the incidence of their occurrence would have been more frequent. However, when these antibodies were demonstrable under the conditions of this experiment, their presence was evidence of a high degree of immunity. In 10 instances both agglutinins and protective antibodies occurred after the same spray; in 9 animals agglutinins appeared before protective antibodies were demonstrable, while in 30 instances protective antibodies occurred without demonstrable agglutinins in the serum. In the 9 rabbits in which agglutinins appeared first, protective antibodies were later demonstrated in 4 instances after the next spraying, in 2 others after 3 additional sprayings, and in 1 rabbit not until after 5 subsequent exposures.

The relative occurrence of agglutinins and of protective antibodies in the serum of rabbits following inhalations of pneumococci is graphically shown in Text-fig. 1. In this figure the mortality curve of rabbits dying from septicemia is also given. It is seen that after the 1st exposure 34 per cent of rabbits died from pneumococcus septicemia. However, no rabbits died from this cause after the 4th spraying. In other words, the more highly susceptible animals were rapidly elimi-



TEXT-FIG. 1. Comparison of mortality, and presence of protective antibodies and agglutinins in rabbits following repeated inhalations of virulent Type I pneumococci.

———— per cent of rabbits dying with pneumococcus septicemia following successive sprayings.

------- per cent of rabbits showing agglutinins in serum following spraying.

— — — per cent of rabbits showing protective antibodies in their serum.

nated by the 1st exposure, while those that survived were either naturally more resistant or had gained some degree of immunity. It is interesting to note that up to the 5th spray an increasing proportion of rabbits developed agglutinins but that after this the percentage remained stationary. On the other hand, with each successive spray an increasing number of rabbits developed protective antibodies until

following the 10th exposure 82 per cent of the total number showed the presence of these immune substances in their serum.

DISCUSSION.

From the foregoing experiments it appears that following repeated inhalations of living Type I pneumococci, rabbits develop a high degree of immunity as evidenced by the presence of demonstrable agglutinins and protective antibodies in their serum. This immunity is probably induced by a few organisms penetrating the respiratory epithelium and entering into the body tissues. It has already been shown that rabbits may even recover from a transient pneumococcus septicemia. The great variations both in the time of first appearance and in the final titre of immune bodies are difficult to explain.

Among the factors which cannot be experimentally controlled are: first, the number of organisms which come to lodge within the respiratory tract following exposure to a bacterial spray; second, the number which after implantation are able to invade the tissues, and third, the final disposition of these bacteria in the animal body. In certain instances the multiplication of the invading organisms goes on unchecked until the death of the animal. In others a transient carrier state may occur with subsequent immunity responses. It is certainly significant that whereas the curve of incidence of protective antibodies steadily increases, the percentage of rabbits showing agglutinins does not materially change after the 5th spraying.

CONCLUSIONS.

1. Following repeated inhalations of Type I pneumococci agglutinins and protective antibodies can be demonstrated in the serum of rabbits.

2. The percentage of rabbits whose serum shows agglutinins remains stationary after the 5th exposure, but the percentage of rabbits showing protective antibodies in their sera steadily rises.

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