PRODUCTION OF IMMUNITY IN MICE BY INHALATION OF PNEUMOCOCCI.

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In preceding papers (1) it has been shown that following inhalation of virulent pneumococci they generally disappear from the lungs of normal mice within a few hours, that infection rarely occurs, and that in only a few instances does death result from a septicemia. The regularity with which pneumococci may be recovered from small pieces of lung is evidence that the organisms enter the lower respiratory tract. Despite the presence of virulent organisms deep in the respiratory tract the animals are only infrequently infected, as evidenced by the large number of survivals. Furthermore, since the organisms are only found in the lungs for a few hours after inhalation there must be an efficient method for their disposal. If mice are first intoxicated with alcohol and then sprayed with pneumococci by the same technique, the pneumococci persist in the lungs for much longer periods and fatal septicemia occurs in as many as 40 per cent of the mice. Evidently alcohol interferes in some way with the mechanism concerned with the disposal of inspired bacteria. No evidence was obtained, however, as to the exact method of this disposal. If the removal of the bacteria is accomplished solely by the action of the ciliary currents, *i.e.* mechanically, one would suspect that no immunity would be acquired, as the respiratory tract is really "outside the body." If, on the other hand, the inspired bacteria penetrate the surface epithelium and actually gain access to the lung tissue proper, some degree of immunity might reasonably be expected to develop.

It is the object of this paper to report certain experiments which show that immunity is present in normal mice which have been repeatedly exposed to an atmosphere containing live virulent pneumo-

cocci in suspension, and to a less extent in mice which have been exposed to an atmosphere containing heat-killed organisms, and further in mice which have survived one or more exposures to a spray of virulent pneumococci while intoxicated.

Method.

The mice were exposed to the living or dead bacteria in the spray chamber previously described (1). The animals were sprayed repeatedly at intervals of from 2 to 3 days with 50 cc. of (1) an 18 hour broth culture of the living virulent organisms, or (2) similar cultures killed by heating at 60°C. for 30 minutes, or (3) washed heat-killed organisms suspended in salt solution.

In the case of intoxicated mice, the alcohol was administered intraperitoneally in 10 per cent solution in saline, 1.5 cc. being given 1 hour before spraying.

The degree of immunity acquired by the mice was measured by the ability of the animal to withstand an intraperitoneal injection of a fatal dose of virulent organisms. These tests were performed 10 days after the last spraying. The survival of mice following an intraperitoneal injection of dilutions of 0.0001, 0.00001, and 0.000001 of an 18 hour broth culture of the homologous organism was used as a measure of the degree of immunity developed in the mice. The virulence of the strain of Type I pneumococcus used throughout was such that a millionth cc. injected intraperitoneally invariably resulted in death in 48 hours. Therefore, the survival of any of the treated mice for a period of 5 days following injection was accepted as proof of an acquired immunity.

EXPERIMENTAL.

It is necessary to emphasize the fact that although the pneumococcus is an organism which generally disappears from the lungs of normal mice within a few hours following inhalation, rarely giving rise to infection, yet, during the course of repeated exposures a fatal invasion of the blood stream will occur in a certain number of mice. In other words, organisms may penetrate the tissues in numbers sufficiently large to produce a fatal infection. Table I shows the number of normal animals which succumbed during the course of spraying with virulent pneumococci.

From the table it is seen that only twenty-nine deaths occurred among 449 normal mice which were repeatedly exposed from one to ten times in gradually decreasing numbers to an atmosphere containing virulent pneumococci in suspension. Unfortunately nine of the animals dying were obtained in a state of postmortem decomposition

such that cultures from them would have been useless, but pneumococci were recovered from the heart's blood of fourteen. It is not possible to state definitely at which exposure infection took place, but in the light of subsequent work it is probable that it occurred at the spray immediately preceding death. The table also brings out the interesting fact that the greatest number of deaths occurred after the second exposure, and that the mortality tended to lessen, until after the tenth exposure no animals succumbed. In other words, those mice which were most susceptible to pneumococcus died early. As will be shown, the surviving mice were those which not

TABLE I.

Fate of Mice Exposed to from One to Ten Inhalations of Pneumococcus Cultures.*

No. of times exposed to virulent pneumococci.	No. of mice exposed.	No. dying.	Pneumococcus recovered from heart's blood.	Pneumococcus not recovered from heart's blood.
1	449	—	—	—
2	446	10	7	3
3	337	4	1	3
4	312	8	4	4
5	211	2	—	2
6	210	1	—	1
7	167	1	1	—
8	165	2	—	2
9	109	1	1	—
10	108	—	—	—
	449	29	14	15

* The discrepancy in the total numbers is due to the fact that some animals were used for intraperitoneal tests or for other experiments.

only had the greatest natural resistance but also had acquired a certain degree of immunity as a result of repeated exposures to the pneumococcus atmosphere.

To demonstrate that a degree of immunity is acquired by mice, even after the second exposure to virulent pneumococci, the following experiment was performed which will serve as an example of other similar ones. 10 days after the second of two exposures to virulent pneumococci the immunity of thirty mice was tested by an intraperitoneal injection of virulent organisms. The results are given in Table II.

From Table II it is seen that six of the thirty animals which had been twice exposed survived an intraperitoneal inoculation of pneumococci greatly in excess of that amount which invariably proved fatal in normal controls. This experiment demonstrates that the resistance of certain animals was increased, although there is a considerable individual variation in this increase.

The degree of immunity acquired by mice which had been exposed more frequently, up to ten times, to an atmosphere containing virulent

TABLE II.

Virulence Test on Thirty Mice Twice Previously Exposed to a Pneumococcus Spray.

Pneumococcus Type I. 0.0001 cc.	0.00001 cc.	0.000001 cc.	Normal controls 0.0001 to 0.000001 cc.
D. 34 hrs.	D. 24 hrs.	D. 28 hrs.	
" 36 "	" 24 "	" 34 "	
" 36 "	" 36 "	" 36 "	
" 36 "	" 48 "	" 36 "	
" 36 "	" 48 "	" 36 "	
" 40 "	" 48 "	" 44 "	D. within 48 hrs.
" 40 "	" 48 "	" 44 "	
" 40 "	" 72 "	S.	
" 44 "	S.	"	
S.	"	"	
Died. 9	8	7	
Survived. 1	2	3	

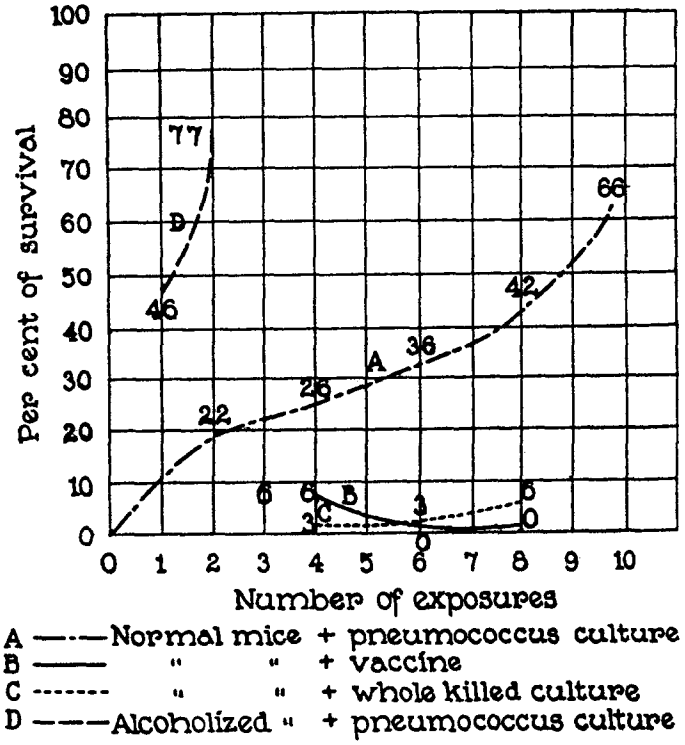
D. indicates died.

S. indicates survival.

The figures represent the number of hours elapsing before the death of the animal.

pneumococci has been tested in like manner. Ten mice in each series were injected intraperitoneally with 0.0001, 0.00001, and 0.000001 cc. of a virulent pneumococcus culture, respectively. The results of these experiments, including the one tabulated above, are given in Text-fig. 1. In this figure the dot and dash line (*A*) represents the results following exposure to virulent living organisms. It is seen that after two exposures 22 per cent of the total number of mice survive an intraperitoneal injection of virulent organisms, while after ten exposures 66 per cent survive.

Since the mice which were naturally of high susceptibility to pneumococcus infection had died early in the course of the immunization, these experiments were performed on mice which possessed a certain natural resistance to the pneumococcus. However, the figures indicate a greater degree of immunity than can be accounted for by mere "selection of the fittest," as is sufficiently evident from the failure



TEXT-FIG. 1.

of any controls to survive (Table II). Furthermore, the gradual increase of acquired protection clearly indicates that repeated exposures to the pneumococcus induce a progressively increasing active immunity. It is thus evident that following inhalation of virulent pneumococci a definite protection is afforded mice even on intraperitoneal inoculation and that the amount of this protection depends to a considerable degree on the number of previous exposures.

In order to determine whether any immunity was produced by the inhalation of dead organisms, mice were sprayed from four to eight times with either whole killed cultures of pneumococcus or dead organisms suspended in saline. The degree of active immunity developed was measured by the intraperitoneal injection of virulent organisms. The results are charted in Text-fig. 1, the dotted line (*C*) representing the results with whole killed cultures and the unbroken line (*B*) those with dead organisms suspended in salt solution. It is seen that exposure to killed organisms likewise produces protection to the intraperitoneal injection of virulent organisms, though one relatively slight as compared with the resistance obtained when living organisms are inspired.

Finally, the degree of acquired immunity in mice which had recovered from the combined administration of alcohol intraperitoneally and pneumococcus spraying was determined. The protection tests in these animals are represented in Text-fig. 1 by a broken line (*D*). The animals which had been exposed to a pneumococcus spray while intoxicated showed a decided immunity which increased in proportion to the number of exposures. Of the mice which had survived one or two preliminary treatments with alcohol and pneumococci, 46 and 77 per cent, respectively, were sufficiently immune to overcome an intraperitoneal injection of virulent organisms. While in this particular instance the increase of immunity is more prompt and slightly greater than in the non-alcoholized animals, the number of mice which survive the treatment itself is very much less and the incidence of infection far greater. Consequently, the animals which successfully survive the preliminary treatment may be considered to possess a high natural resistance. However, the great degree of immunity exhibited by these mice can hardly be accounted for by the fact that the more susceptible had died as a result of exposure to a pneumococcus atmosphere while intoxicated. Indeed, since the pneumococci persist much longer in the lungs of intoxicated mice, it seems not unlikely that the noteworthy increase in the immunity of the animals is due to a greater and more rapid development of antibodies.

DISCUSSION.

The results obtained by Webster (2) on microbic virulence and host susceptibility in mouse typhoid infection are strikingly paralleled by the results of the present work with pneumococci. This author found that if he fed 100 mice 6,000,000 typhoid bacilli by stomach tube, approximately 70 died with the organism in the blood and stools, and 30 survived. In 20 of the latter typhoid bacilli were present in cultures from the stools only during the first few days and not in cultures from the blood,—in which respect these animals were naturally immune,—while in the remaining 10 animals the organisms persisted in the stool sometimes as long as 6 months and were temporarily present in the blood stream. Such animals recovered and developed agglutinins. The second group mentioned represent animals which develop an acquired immunity after passing through the so called "carrier state" or condition of "latent infection."

Cecil and Steffen (3) were able to show that intratracheal injections of killed cultures rendered monkeys insusceptible to later intratracheal injections of living pneumococci. They were unable, however, to produce any immunity by spraying killed cultures into the pharynx. Although Jones (4) could immunize rabbits against *Bacillus avisepticus* by the intratracheal injection of killed cultures, he was unable by means of sprays of heat-killed organisms to increase the resistance appreciably. He concluded that in the latter instance insufficient antigen was absorbed to afford measurable protection against subsequent intratracheal infection.

From the foregoing experiments it appears that a high degree of active immunity is produced in mice following repeated inhalations of living pneumococci, and that a less marked increase in resistance is afforded animals exposed to a spray of dead organisms. To explain this acquired protection, one is forced to assume that the inhaled bacteria or bacterial cells actually gain access to the body tissue; that is, they are not only implanted on the mucosa of the lower respiratory tract, but actually penetrate the respiratory epithelium. Very few living organisms probably gain entrance at any one time. In fact, I have never observed pneumococci in histological sections from the animals, while even culturally it is not sufficient merely to

inoculate a blood agar plate with the cut surface of the lung tissue, for so few are the organisms present that sections of the tissue itself must be cultured in broth. Even under these optimum conditions growth is not infrequently delayed until after 24 hours. One may suppose the high degree of active immunity which results from the inhalation of live organisms to be due to the limited multiplication of the organisms within the tissues. The possibility exists, however, that actual multiplication of the pneumococci does not occur but that mice may be immunized to a fairly high degree by the repeated penetration of a very few virulent organisms which are quickly destroyed.

CONCLUSIONS.

1. A definite degree of active immunity can be induced in mice through the repeated inhalation of live pneumococci.

2. Only slight immunity is induced in mice by the repeated inhalation of killed organisms.

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