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INHALATION EXPERIMENTS ON MICE WITH PNEUMOCOCCI.

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THE purpose of the experiments recorded in this paper is to test the resistance of immunised mice to infection by inhalation, to attempt the production of a local reaction in the lungs, and to make observations on the carrier state in immune and non-immune mice.

The production of fatal infection in mice by spraying with cultures of pneumococci has been found by most observers to be difficult, even though cultures have been used which were highly virulent by other channels. Stillman and Branch[1], for example, have studied recently the pulmonary method of infection by spraying and state that positive results were infrequent when normal mice were used, but the pneumococci persisted for a much longer period in the lungs, and general invasion was of much more frequent occurrence in mice which had been rendered unconscious by an intraperitoneal dose of alcohol. He found that in his inhalation experiments less than 3 per cent. of normal mice died of pneumococcal septicaemia, while over 40 per cent. of alcoholised mice succumbed to blood infection. He believed that the normal mechanism of disposal of inspired bacteria was interfered with by the administration of alcohol, though his experiments yielded no evidence as to how alcoholic intoxication rendered the lungs more permeable. Various methods of lowering the resistance of the mice after spraying with pneumococci, such as sudden chilling and exposure to cold, did not increase the numbers of general infections.

Before the results of Stillman's experiments were published, I had already made a number of inhalation experiments with cultures of Types I and II pneumococci on mice which had been immunised in various ways, and had obtained a high percentage of fatal infections among the normal controls. My method of spraying was similar to that adopted by Stillman, but no special efforts were made either to alter the permeability of the mucous membranes or to lower the systemic resistance.

Additional experiments have been performed by me since the publication of Stillman's paper and a test has been made of the method of alcoholisation suggested by him. It would appear that the important conditions for the production of general infection by spraying are probably (1) high invasive qualities of the cultures used, and (2) the introduction of a sufficient number

[1] Stillman, E. G. and Branch, A. (1924). Experimental production of Pneumococcus pneumonia in mice by the inhalation method. *Journ. Exp. Med.* XL. 733–752.

of the bacteria into the deeper air passages. The action of alcohol, where it increased the percentage of successful infections, might be explained on the assumption that the unconscious state of the mouse permitted a larger dose of pneumococci to penetrate to the bronchioles and lung alveoli without postulating any lowering of systemic resistance. I sprayed the mice in batches of not more than 12 or 15 in a large glass jar. The pneumococcus cultures were grown in trypsinised meat broth and were turned into a very fine mist by means of a hand nebuliser. The mice were exposed to the spray for about 15 minutes, during which 15 c.c. of broth culture were vaporised. At the end of the 15 minutes' spraying, the mice were removed from the jar to wire cages.

It may be noted here that the later spraying experiments were less successful than the earlier ones, a result which I attribute to a mechanical defect, viz. the gradual blocking of the air channel of the nebuliser, so that the force was not sufficient to produce as fine a spray as before.

Description of Experiments.

Experiment 1.

In this experiment there were 13 immunised mice and 10 normal mice. The protected mice had been treated with a heated vaccine containing both Types I and II cultures, and had survived an intraperitoneal test dose of 0·0001 c.c. of Type I culture 14 days before the present spraying experiment. The broth culture used for spraying contained in equal parts the Types I and II cultures which have been used for testing immunity throughout these experiments.

All but one of the ten control mice succumbed to pneumococcal septicaemia. Eight were dead within 3 days, the blood infection being due in seven cases to Type I and in one to Type II. The ninth mouse died 11 days after spraying and a culture of Type II was grown from the blood.

Of the 13 protected mice two died in 3 and 4 days respectively from Type I septicaemia. The remainder survived, being kept under observation for 14 days. These results show (1) that, by using highly virulent cultures and a fine spray, pneumococcal septicaemia can be readily produced in mice by inhalation, without recourse to any method of lowering resistance, and (2) that vaccination either subcutaneously or intraperitoneally confers immunity against infection by inhalation.

The fact that in the case of two mice it was possible to overcome by spraying an immunity which had been proof against an intraperitoneal inoculation shows that the numbers of pneumococci inhaled must have been considerable.

Experiment 2.

This experiment was made to ascertain whether passive immunity produced by a preliminary injection of antipneumococcal serum would be as effective as vaccination in protecting against infection by inhalation. Thirteen

mice received each a dose of 0·2 c.c. of Type I serum intraperitoneally and were then sprayed with broth culture of Type I; 13 normal mice were sprayed as controls.

All the 13 protected mice survived, while eight of the control mice died of pneumococcal septicaemia after periods ranging from 2 to 6 days. The mice were kept under observation for 3 weeks, as it had been found from a preliminary experiment that mice which have been sprayed with culture may harbour the pneumococcus for 11 and 15 days, finally succumbing to a pneumococcal septicaemia. In this experiment all the remaining mice, both normal and protected, survived the period of 3 weeks. The fatality rate among the normal controls was not so high as in the first experiment, although the same virulent Type I strain was used. It is clear, however, that the intraperitoneal injection of protective serum raises the resistance of mice to general infection with pneumococci through inhalation.

At the end of the period of observation, that is, 3 weeks after the injection of serum, the immune batch of mice was again sprayed with Type I culture. One out of eleven (two had succumbed to intercurrent disease) developed pneumococcal septicaemia, a result which might indicate that the effect of the serum was tending to disappear.

One of the objects of these spraying experiments was to determine whether the air passages of the mice which survived became free from the inspired pneumococci, or whether the mice became chronic carriers. The latter condition was suspected from the observation referred to above, that mice may remain well for periods up to 15 days after spraying and then die of pneumococcal septicaemia.

The following method was used to demonstrate the presence of pneumococci in the nasopharynx. The mouse was killed with chloroform and the neck was seared; the trachea was cut across and thrown forward so that the posterior nares were revealed. The mucous membrane together with bits of bone was scraped away with the point of a knife and placed in a small tube of blood broth which was incubated at 37° C. for a few hours. This culture was then plated and was generally also inoculated into a mouse. The results were often unsatisfactory, owing to the presence of Gram-negative bacilli which overgrew the plate cultures and killed the mice before any pneumococci which might have been present had time to grow. Success in obtaining pneumococci was therefore largely a matter of chance. The method was varied on one occasion; the nasopharynx was washed with a little broth and the washings were used for culture and inoculation; the results, however, were not so good as by the dry method.

Among the five surviving control mice which were killed 3 weeks after spraying, one was found to be a nasopharyngeal carrier of Type I pneumococci. The immunised mice were killed 3 weeks after the second spraying; no pneumococci could be demonstrated either in the nasopharynx or in the lungs.

Experiment 3.

From a practical point of view it is of interest to know whether vaccination with Types I and II will afford protection against infection by inhalation with the heterogeneous strains of Group IV. In the first experiment described above, 10 mice which had been vaccinated with a mixture of Types I and II cultures were sprayed with the same virulent types and resisted infection. After being observed for 14 days they were again sprayed with a mixed culture consisting of Type III and two Group IV strains. Five normal mice served as controls. At the end of a week, two of the control mice had died of pneumococcal septicaemia, one infected with Type III and the other with a Group IV strain, while all the immunised mice were well. The latter were re-sprayed with the same cultures together with three fresh mice and the three surviving controls. Again all the immunised mice survived while two of the controls died, one infected with Type III and the other with one of the Group IV strains.

Although the experiment is only on a small scale, it does show that active immunisation with the chief types I and II will protect mice against the heterologous strains of Group IV and Type III when the test of immunity is made by the inhalation method.

The surviving mice in this experiment, 10 immunised and 4 controls, were killed 22 days after the last spraying and were examined to discover whether any pneumococci still persisted in the nasopharynx and lungs. Each tissue was cultured and then injected into mice in the manner described above. No pneumococci could be demonstrated in the immunised mice, but one of the control mice was found to be harbouring in the nasopharynx one of the Group IV strains.

Experiment 4.

This was a test of passively immunised mice by inhalation with a mixed culture of Types I and II.

Two series of mice were immunised with protective sera; 18 mice received 0·2 c.c. of Type I serum and 19 mice the same amount of Type II serum, each injected intraperitoneally. On the same day the two sets of mice were exposed for about 15 minutes in a large glass jar to a fine spray of pneumococcal broth culture which consisted of a mixture in equal parts of Types I and II, after which they were distributed in small wire cages. The results are summarised in Table I.

Out of the 18 mice immunised with Type I serum eight died after periods varying from 3 to 9 days and in every case a Type II culture was grown from the blood. On the 11th day after the injection of serum a mouse died of Type I infection. The remaining nine mice appeared well and cultures made from the tail blood were negative. They were all killed on the 13th day of the experiment and their lungs and nasopharynges were examined for the presence of pneumococci. Pieces of the lungs were removed and

scrapings were taken from the posterior part of the nasopharynx after dissection; direct plates were made and also subcultures in small quantities of blood broth. It was impossible to avoid contamination from the palate and the chief difficulty was the rapid growth of Gram-negative bacilli which were virulent when inoculated into the mouse. From four of the nine apparently healthy

Table I. *Mice sprayed with a mixture of Types I and II cultures.*

No. of mouse	Result	Pneumococcus culture obtained: Type	Pneumococcus culture obtained: Source
	Series 1 immunised with Type I serum		
1	Died 3 days	II	Blood
2	,, 4 ,,	II	,,
3	,, 4 ,,	II	,,
4	,, 4 ,,	II	,,
5	,, 5 ,,	II	,,
6	,, 8 ,,	II	,,
7	,, 9 ,,	II	,,
8	,, 10 ,,	II	,,
9	,, 11 ,,	I	,,
10	,, 13 ,,	No pn.	,,
11	Killed 13 ,,	No pn.	Lung and nasopharynx
12	,, 13 ,,	I	Nasopharynx
13	,, 13 ,,	II	Lung
14	,, 13 ,,	No pn.	Lung and nasopharynx
15	,, 13 ,,	I	Nasopharynx
16	,, 13 ,,	No pn.	Lung and nasopharynx
17	,, 13 ,,	I	Nasopharynx
18	,, 13 ,,	No pn.	Lung and nasopharynx
	Series 2 immunised with Type II serum		
19	Died 3 days	I	Blood
20	,, 3 ,,	I	,,
21	,, 3 ,,	I	,,
22	,, 3 ,,	I	,,
23	,, 3 ,,	I	,,
24	,, 3 ,,	I	,,
25	,, 3 ,,	I	,,
26	,, 3 ,,	I	,,
27	,, 3 ,,	I	,,
28	,, 4 ,,	I	,,
29	Killed 14 ,,	No pn.	Lung and nasopharynx
30	,, 14 ,,	I	Lung
31	,, 14 ,,	I	Nasopharynx
32	,, 17 ,,	No pn.	Lung and nasopharynx
33	,, 17 ,,	,,	,, ,,
34	,, 17 ,,	,,	,,
35	,, 19 ,,	,,	,, ,,
36	,, 19 ,,	,,	,, ,,
37	,, 19 ,,	I	Nasopharynx

mice cultures of pneumococci were recovered; one was Type II and was obtained from the lung, and in three cases Type I cultures were obtained from the nasopharynx.

Of the 19 mice immunised with Type II serum ten died of pneumococcal septicaemia, all, with one exception, within 3 days after spraying. In each case a Type I culture was grown from the blood. The remaining nine mice were killed 14 days to 19 days after spraying and were apparently healthy. A Type I culture was grown from the nasopharynx of one mouse and from the lung of another; from the rest no pneumococci could be obtained.

The first point of interest in this experiment is that about half the mice from each series succumbed to invasion of the body with pneumococci, presumably through some mucous membrane or through the lung alveoli. The remaining half resisted invasion by the type in the mixed culture against which they were not specifically protected. That the culture had penetrated into the air passages is clear from the subsequent nasopharyngeal cultures. One cannot, however, conclude that the serum injection had raised the resistance against the heterologous strain, because it would not have been an unexpected result if half of a series of normal mice had failed to become generally infected after spraying. No mouse protected with Type II serum succumbed to invasion with Type II pneumococci, and Type I serum afforded almost complete protection against invasion with Type I. There was one exception in the Type I protected series; a mouse died of Type I septicaemia 11 days from the beginning of the experiment. It appears, as might be expected, that the immunity conferred by the serum had disappeared from this mouse through the lapse of time. I have tested at intervals mice injected with serum and find that there is a fairly rapid diminution in their resisting powers. Immediately after immunisation an intraperitoneal dose of 0·1 c.c. of culture was resisted; on succeeding days the lethal dose was smaller, until on the 8th day the mice failed to resist 0·001 c.c. of culture.

The duration of life was more irregular in the Type I protected series, the Type II culture appearing to set up a more chronic infection than the Type I. Two of the mice dying after 5 and 8 days respectively from Type II infection had solid lungs and the pleural cavities were filled with fluid. On the other hand the mouse in the same series which succumbed to Type I infection 11 days after spraying showed no evidence of local infection of the lungs.

The occurrence in mice of typical pneumonic consolidation is an interesting feature of these inhalation experiments. The penetration into the tissues of a mouse by pneumococci generally results in acute septicaemia and it is only in special circumstances that a local inflammatory reaction, such as pneumonia, follows. In the two instances mentioned above the injection of Type I serum may have produced, against the heterologous Type II strain, a slight degree of immunity which was sufficient to enable the lung tissues to offer a local resistance[1]. On another occasion typical pneumonia was set up in a non-immune mouse by a culture which probably had low invasive qualities. This appeared to be the reason since out of the six mice sprayed two died after a delay of 6 days and one in 8 days. In one of the former the right lower lobe of the lung was grey and solid.

In the second series of mice immunised with Type II serum all the fatal infections with Type I were acute and there was no instance of consolidation of the lungs.

[1] Stillman and Branch (*loc. cit.* p. 733) have made experiments which show that this is feasible (see footnote, p. 1).

The subsequent examination of the surviving mice in both the above series provided ample confirmation of the observation made in the previous experiment that mice may harbour virulent pneumococci without apparent ill effects. The carrier condition persisted in one mouse for 19 days after spraying. Of the two types Type I was found more often in spite of the fact that it was apparently the more virulent. Since the Type I carriers were equally distributed in both series, there was no evidence that the state of passive immunisation had any influence as regards the persistence on the mucous membrane of any particular type. It has already been shown in a previous experiment that normal mice may carry virulent pneumococci and may become invaded after so long a period as 13 days after spraying.

Experiment 5.

The following spraying experiment was undertaken mainly to compare the persistence of virulent pneumococci in the air passages of normal mice and in those of mice which had been immunised, some actively and some passively. There were two series of immune mice, one protected against Type I and the other against Type II, and the culture used for spraying contained both

Table II. *Mice sprayed on three occasions with a mixture of Types I and II cultures.*

	1st spraying 31. xii. 24		2nd spraying 9. i. 25		3rd spraying 15. i. 25		
Number of Mice	Died of Pn. Septicaemia	Type of culture from blood	Died of Pn. Septicaemia	Type of culture from blood	Died of Pn. Septicaemia	Type of culture from blood	Observations on the carriers of pneumococci amongst the survivo
9 normal	1 died, 5 days (lung solid)	II	None	—	1 died, 4 days	II	7 killed 4 to 11 days after la spraying. One mouse (11 day carried Type I.
6 alcoholised	1 died, 5 days	II	None	—	None	—	One died next day aft spraying; pn. in lung but n in blood. No carriers among the rest 3 months later
10 immunised with Type I serum	1 died, 5 days	II	1 died, 3 days	II	None	—	8 killed 4 to 11 days. O mouse (5 days) carried Type in lungs
9 immunised with Type II serum	1 died, 3 days; 1 died 5 days	I I	1 died, 4 days; 1 died, 4 days	I I	1 died, 5 days	I	No carriers amongst the su vivors killed 5 days later
13 vaccinated with Type I culture	None	—	1 died, 3 days	II	1 died, 6 days	II	No carriers found
10 vaccinated with Type II culture	1 died, 6 days (lung solid)	I	None	—	1 died, 5 days	I	One killed 4 days was a nas pharyngeal carrier of Type One killed 99 days carri Type I in the nasopharynx

Types I and II. There was thus a possibility of showing whether the state of immunity of the mouse would have any influence in determining which of the two types in the mixed culture was more speedily eliminated from the mucous membrane of the nasopharynx and the lung alveoli. There was no indication, it may be remarked, in the fourth experiment that a preliminary injection of serum had any influence on the carrier condition.

In addition to the normal controls there were six non-immune mice which had received a preliminary intraperitoneal injection of 1·5 c.c. of 10 per cent. alcohol and were unconscious during the spraying. Stillman, as mentioned earlier, found that mice in that condition more frequently succumbed to general infection after inhaling pneumococci than did normal mice. The actual spraying was carried out as in the preceding experiments; but it was found afterwards that the nebuliser was not producing so fine a spray, and to this fact is probably to be attributed the relatively low incidence of infection. The above table (Table II) shows the details and results of the experiment.

Discussion of results of Experiment 5.

The six alcoholised mice were stupefied only on the occasion of the first spraying, and one out of six died as compared with one out of nine normal mice. This small experiment does not furnish any evidence that the susceptibility of mice to general invasion with pneumococci after spraying is increased by alcoholisation.

The immunised mice, excepting one series, were fatally infected with the pneumococcus type against which they were not specifically protected in about the same proportion as the non-immune mice. In the exceptional series the mice protected with Type II serum showed a high proportion of fatal Type I infections (five out of a total of nine) a result which must be attributed to accidental circumstances. None of the mice succumbed to infection with the type against which they were either passively or actively immunised.

The chief object of the experiment was to investigate the carrier condition of the surviving mice. I have already shown that virulent pneumococci which are capable of killing the mouse in minute doses when introduced into the body can persist for considerable periods on the nasopharyngeal mucous membrane without ill effects. This observation appeared to hold out promise of obtaining useful information upon the influence of vaccination on chronic carriers of respiratory organisms. In this preliminary experiment it was hoped to show by spraying mice with a mixed culture of Types I and II which of the two types would be more likely to persist in a mouse immunised against one of them. Both actively and passively immunised mice were used in order to see whether elimination of the inhaled pneumococci depended on the presence of antibacterial substances in the blood or on the actively immunised cells. The number of instances in which pneumococci were obtained were, however, too few in number for any conclusions to be drawn. The demonstration of pneumococci in the nasopharynx of a mouse is uncertain and the technique must be improved before one can obtain evidence of statistical value. Since there are always other organisms present in the nasopharynx, it is necessary to devise a method in which the pneumococci present will outstrip the former in their growth. The great difficulty is the frequent presence of Gram-negative bacilli which grow rapidly and are more virulent for mice when inoculated than the pneumococcus.

The surviving mice were killed at different periods after spraying, the earliest being 4 days and the latest 3 months. One may note that there were two carriers of Type I among the mice vaccinated with Type II culture, one persisting for 99 days after spraying, while there were no carriers of Type I among the Type I vaccinated mice.

No carriers of Type II were found in any of the series.

Experiment 6.

This experiment was made to test the following method of taking cultures from the nasopharynx. The posterior nares were exposed as before from behind and a tiny ball of dry sterile wool was applied to the mucous membrane around the aperture. The swab was rubbed on a fresh blood agar plate and then dropped into a tube of blood broth. A second ball of wool was inserted into the aperture of the posterior nares with a fine pair of forceps and cultures from it were made as before. This method gave good results, as will be seen from the account of the experiment.

(*a*) Four mice were sprayed with Type I culture. One mouse died in 3 days of pneumococcal septicaemia, a second was killed 12 days after spraying; the nasopharyngeal plates were overgrown with Gram-negative bacilli. The third and fourth mice were killed after 16 days and in both cases the plates from the direct swabs yielded pneumococcus colonies.

(*b*) Four mice were sprayed with Type II culture. They were killed as follows: No. 1, 12 days after spraying; pneumococci were not found. Nos. 2, 3 and 4, 13 days after spraying; Type II pneumococci were grown from the direct swabs from the nasopharynx of each of the three mice.

In one case three out of four mice and in the other two out of four mice were found to be carriers of pneumococci in the nasopharynx 13–16 days after spraying. On some plates the pneumococcus colonies were in the majority. On plates made with chloroformed blood the smooth shiny colonies of the virulent pneumococcus are readily picked out from the common nasopharyngeal organisms. The rough form of the pneumococcus has not been found and its identification would be extremely difficult.

SUMMARY OF RESULTS.

Fatal general infection can be readily produced in mice by spraying with pneumococcal culture in a closed chamber without resorting to any artificial means of lowering the animal's resistance.

In the experiments recorded the proportion of unprotected mice which developed septicaemia varied considerably, the highest being nine out of ten. These irregularities may be partly attributable to variations in (1) the fineness of the spray, and (2) the virulence of the culture.

In unprotected mice death from septicaemia rarely occurs before the 3rd day after spraying and may be delayed for a week or more.

Vaccination by subcutaneous and intraperitoneal inoculation with heated

cultures afforded protection against infection by inhalation to 11 out of 13 mice. Two succumbed to septicaemia although they had resisted 14 days earlier an intraperitoneal dose of 0·0001 c.c. of culture. The culture used for spraying was evidently in a highly virulent state since nine out of the ten controls died of septicaemia.

The above experiments were with homologous cultures but tests have also been made of the efficacy of vaccination against heterologous strains. For example, vaccination with Types I and II cultures gave apparently a certain amount of protection against infection by spraying with a mixture of Type III and Group IV strains.

Passive immunity induced by an injection of antipneumococcal serum immediately before spraying was effective against the homologous types in all of a batch of 13 mice. The resistance showed a tendency to diminish with the lapse of time, since on re-spraying the same lot of mice 3 weeks later one mouse developed septicaemia.

In unprotected mice where death was delayed for some days after spraying there was generally no evidence to the naked eye of any local pulmonary lesions. Rarely typical grey consolidation of portions of the lungs was found. A pneumonic condition occurred more frequently in mice in which a slight degree of immunity had been produced. For example, a Type II pneumonia was found in a mouse which had been immunised against Type I.

The production of the carrier state is a regular sequel to spraying even with the most virulent cultures of pneumococci. A large proportion of the mice which survived spraying were nasopharyngeal carriers a fortnight later and the condition persisted in one mouse for 99 days. A carrier of pneumococci may succumb to septicaemia 15 days after the date of infection; it is not known whether any immunity develops in carriers.

The presence, about the pharynx of mice, of Gram-negative bacilli which grow rapidly in culture and are of high virulence when introduced parenterally is a source of difficulty in attempts to demonstrate pneumococci.

An attempt was made to ascertain whether, after spraying, nasopharyngeal carriers of pneumococci were more numerous among normal or among protected mice. The results were inconclusive, but there was no marked indication in the experiments recorded that the state of immunity influenced the carrier condition.

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