STUDIES ON IMMUNOLOGICAL RELATIONSHIPS AMONG THE PNEUMOCOCCI.

II. A Comparison of the Antibody Responses of Mice and of Rabbits to Immunization with Typical Type III Pneumococci and to Immunization with a Related Strain.

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INTRODUCTION.

The previous paper (1) dealt with the properties of a mouse-virulent strain (Thomas)[1] of Pneumococcus, which is immunologically related to, but not identical with typical Type III pneumococci. One of its most prominent properties is its antigenic effectiveness in rabbits. In contrast to the rarity of a detectable type-specific (anti-S)[2] response on injection of typical Type III strains, immunization with the Thomas strain invariably yielded high titres of anti-Thomas antibodies and usually also yielded measurable amounts of antibodies specifically[2] reactive with Type III pneumococci. These differences between the

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[1] The terms "Thomas" strain and "anti-Thomas" serum are employed, as in the preceding paper, to refer to the "non-typical" strain and its antiserum.

[2] The distinctions between the anti-S antibody and the anti-P antibody in the serological reactions of Type III pneumococci have been pointed out in detail by Tillett (3). As stated in the preceding paper, the immunity systems involved in the reactions of anti-Thomas serum with typical Type III pneumococci are the same or similar to the S-anti-S systems involved in the type-specific reactions of the "fixed" types with their homologous antisera. The anti-Thomas serum is just as devoid of effect upon Types I and II pneumococci as is anti-Type III serum, and the Thomas bacteria are just as little affected by anti-Type I or anti-Type II serum as are typical Type III pneumococci.

response of rabbits to the Thomas strain and their response to typical Type III strains seem to be important, especially in regard to the anti-Type III response elicited by injection of the Thomas strain.

It has been general experience (2), not only with rabbits but also with larger animals, that potent anti-Type III immune sera are more difficult to obtain than are potent anti-Type I and anti-Type II immune sera. The question arises, therefore, whether the antigen which gives rise to the Type III anti-S antibody is intrinsically a less effective antigen in all animals, or whether this antigen which seems to be so ineffective might not in other animals elicit responses comparable to those invoked by other types of pneumococcus anti-S antigens.

As a step in the accumulation of data on this question, we have compared the antibody response invoked in rabbits with that invoked in mice when the two species of animals are immunized with typical Type III pneumococci and with the related, but different, Thomas strain. The measurements of the immunity response have included antibodies reactive with the Thomas strain as well as those reactive with typical Type III strains, for anti-Type III horse serum and anti-Thomas rabbit serum usually contain antibodies reactive with both kinds of pneumococci (Thomas and Type III).

EXPERIMENTAL.

Comparison of the Antibody Response of Rabbits to Immunization with Typical Type III Pneumococci with their Response to Immunization with the Thomas Strain When the Amounts of Bacterial Antigen Are Equal.

The fact that rabbits respond poorly to Type III pneumococci is clearly shown in Tillett's (3) extensive study in which 18 out of 28 rabbits failed to produce in their serum even the small amount of type-specific antibody detectable by the delicate mouse protection test. That the Thomas strain is more effective is evident from the results of the preceding investigation (1) in which, in addition to the uniformly high degree of anti-Thomas potency in all sera, only 1 out of 10 rabbits failed to produce the small amount of antibody required to specifically protect mice against virulent Type III pneumococci. These facts strongly suggest that the Thomas bacteria are more effec-

tive in invoking an anti-Type III response in rabbits than are the typical Type III bacteria themselves. It seemed desirable, however,

TABLE I.

Comparison of the Response of Rabbits to the Injection of Typical Type III Pneumococci with the Response to Equal Amounts of the Thomas Strain.

Immune serum		Anti-Type III potency									
		Agglutination						Passive protection of mice *vs.* virulent Type III culture			
		Heated broth culture			Concentrated suspension of heated bacterial cells						
Rabbit	Immunization material	Serum dilution			Serum dilution			Dose of culture, *cc.*			
		Undiluted	2/5	1/5	Undiluted	1/5	1/10	1×10^{-3}	1×10^{-4}	1×10^{-5}	1×10^{-6}
1	Thomas strain	++	+	0	fff	ff	f	D	S	S	S
2	Thomas strain	0	0	0	f	0	0	D	D–S	S	S
3	Typical Type III strain	0	0	0	0	0	0	D	D	D	D
4	Typical Type III strain	0	0	0	0	0	0	D	D	D	D
Anti-Type III horse serum (control)		+++	+++	+++	fff	fff	fff	D–S	S	S	S
Anti-Type I and anti-Type II horse serum (controls)		0	0	0	0	0	0	D	D	D	D

+++ = compact disc, with clear supernatant, after 2 hours at 37°C.; ++ = compact disc, but supernatant not entirely clear; + = definite granulation of the bacteria which persists after thorough shaking.

fff = sedimentation of agglutinated particles almost complete within 10 minutes at 25°C.; ff = sedimentation begun and fluid partially cleared by aggregation of agglutinated particles; f = definite granulation of the bacteria but fluid not cleared by aggregation.

D = mice died within 72 hours in all tests; D–S = protection irregular, mice dying in some tests and surviving in others; S = mice survived in all tests. Virulence controls died within 72 hours when injected with 1×10^{-8} cc. of culture.

that a comparison be made by experiments in which the amount of bacterial antigen and the number of injections were exactly the same.

Two rabbits were immunized against typical Type III pneumococci by two courses of six daily injections of heat-killed bacterial suspension equivalent to 5 cc.

of broth culture in each dose; a week's rest was given between the first and second courses, and the animals were bled 10 days after the last injection. Two other rabbits of the same weight were immunized with the Thomas strain by the same number of injections and with the same amount of bacterial suspension. A summary of the tests of the immune sera is presented in Table I.

The results in Table I show that the particular rabbits injected with typical Type III pneumococci in this experiment failed to produce antibodies reactive with the homologous bacteria, although both of the rabbits immunized with the same amount of the related but different strain, produced antibodies reactive with typical Type III pneumococci. While the anti-Type III potency of the anti-Type III and anti-Thomas serum as obtained from different immune rabbits will vary, the individual rabbits of each series used in this particular experiment yielded serum that can be accepted as possessing about the average potency exhibited by Tillett's series of 28 rabbits and by our previous series of 10 rabbits. Hence, with the above results considered as representative of those most likely to be obtained with rabbits, it is evident that rabbits can produce antibodies specifically reactive with typical Type III pneumococci more readily when injected with the Thomas strain than when injected with typical Type III pneumococci themselves. The results in Table I are important as evidence that this relation holds true when the amount of total bacterial antigen and the conditions of immunization are kept constant.

Both of the rabbits injected with the Thomas bacteria yielded serum potent against the homologous strain, while neither of the anti-Type III rabbit sera reacted at all with the Thomas strain. Since our chief interest is in the anti-Type III response, the results of the tests of the anti-Thomas potency are not included in Table I.

Comparison of the Active Immunity Response of Mice to Vaccination with Typical Type III Pneumococci with the Response to Vaccination with the Thomas Strain of Pneumococci.

The preceding experiment dealt with the responses of rabbits to immunization with typical Type III pneumococci and to immunization with the Thomas strain. The following experiment deals with the responses of mice to immunization with these two related but different kinds of pneumococci.

Immunization.—Two series of 60 white mice, about 3 months old, were selected for immunization; one series was injected with the Thomas strain and the other with a typical Type III strain. The organisms from 10 hour broth cultures were resuspended in salt solution and heated at 60°C. for 30 minutes. The suspensions of the two kinds of pneumococci were compared and when not approximately equal in turbidity, the stronger suspension was diluted until the concentration of total bacterial substance in each was about the same. Each mouse received, subcutaneously, 0.5 cc. of the respective vaccine (Type III or Thomas), equivalent to 0.15 cc. of broth culture, every 2 days for six doses; after which time one dose was given intraperitoneally. 10 days after the last injection, 20 mice from each series were tested for immunity against the two kinds of pneumococci. The remainder were given a second course of immunization with freshly prepared vaccine in the same doses as in the first course. After a rest period of 10 days, another lot of the immunized mice was tested for immunity either against the Thomas strain or against the typical Type III strain.

Tests of Immunity.—The active immunity of the vaccinated mice was tested by intraperitoneal injection of 0.5 cc. of broth containing the desired amount of a 10 hour broth culture of virulent pneumococci. Some of the mice vaccinated with typical Type III were tested against the Thomas strain and others against the homologous typical Type III strain; the Thomas-vaccinated mice were likewise tested against the typical Type III strain as well as against the homologous strain.

The doses used were 1×10^{-5} and 1×10^{-6} cc. of broth culture. These doses are not unreasonably small since both cultures were highly virulent and killed mice regularly in doses of 1×10^{-8} cc. Moreover, it seemed unwise to overtax the active immunity of the mice, especially in the case of Type III, in which the passive immunity conferred by immune horse serum is usually overwhelmed by doses above 1×10^{-4} cc. As a matter of fact, however, the Type III-vaccinated mice which had responded at all, apparently were able to resist doses approximately equivalent to those against which Type III horse serum can protect, for 3 out of 4 vaccinated mice which were tested against 1×10^{-4} cc. resisted this large dose in a later experiment.

The tests were made after both one and two courses of vaccinations. However, one course proved as effective as two courses, and since there is no essential difference in the tests, the results of the entire experiment are summarized in Table II.

Table II presents a summary of the results of experiments upon the active immunity response of mice to vaccination with the two related, but different, kinds of pneumococci—*i.e.*, a typical Type III strain and the Thomas strain. In a previous investigation (4) on the active immunization of mice against Type II pneumococci, it was found that within the zone of reasonably small dosage, the number of invading bacteria was a relatively unimportant factor in com-

parison to the factor of the differences in the immunity responses of the individual mice. For this reason, we believe the results of experiments on the active immunity response of mice can best be analyzed upon the basis of the percentage of individuals protected among a large group of vaccinated mice.

In the analysis of Table II, it is best to consider first the response of the mice as indicated by their immunity against the same strain as that with which they were vaccinated. It is evident that the mice vaccinated with Type III pneumococci responded fairly well in respect to the development of an immunity affording homologous protection, for 75 per cent of the 20 animals tested survived the injection

TABLE II.

Summary of Tests of the Response of Mice to Immunization with the Typical Type III Strain and with the Thomas Strain as Evidenced by Their Active Immunity against Virulent Cultures of These Two Kinds of Pneumococci.

Vaccinated mice	Mice vaccinated with typical Type III pneumococci		Mice vaccinated with Thomas strain of pneumococci	
	Homologous protection (*vs.* 1×10^{-5} or 1×10^{-6} cc. of virulent Type III culture)	Heterologous protection (*vs.* 1×10^{-5} or 1×10^{-6} cc. of virulent Thomas culture)	Homologous protection (*vs.* 1×10^{-5} or 1×10^{-6} cc. of virulent Thomas culture)	Heterologous protection (*vs.* 1×10^{-5} or 1×10^{-6} cc. of virulent Type III culture)
Number tested	20	15	22	15
Number protected	15	0	9	0
Percentage of protection	75	0	40	0

of virulent Type III bacteria. The response of the mice to the Thomas vaccination was apparently somewhat less effective, for only 40 per cent of the animals tested survived the injection of the Thomas or homologous bacteria. Thus, it is evident that from the standpoint of the development of immunity against the homologous kind of pneumococci, mice differ from rabbits in respect to the relative effectiveness of the typical Type III antigen and the Thomas antigen. Rabbits almost invariably respond well to the Thomas strain by producing immune sera of a high degree of potency, comparable in all respects to that elicited by Type II pneumococci, while an effective response of rabbits to the typical Type III antigen is the excep-

tion rather than the rule. The important fact in regard to the homologous protection in Table II is not that the mice do not respond uniformly well to the Thomas vaccination, but rather that such a large percentage of the mice are effectively protected by the Type III vaccination. The percentage of homologous protection exhibited by the Thomas-vaccinated group is just as high as that obtained in a previous investigation with Type II pneumococci, in which only 30 to 40 per cent of a large number of mice were effectively protected by homologous vaccination by the procedure employed in the present study. Thus, it appears that in respect to the development of homologous protection, mice respond just as well, and even better, to the Type III antigen than they do to two other kinds of pneumococci which in rabbits invoke incomparably better responses than do Type III pneumococci.

The response of the mice as evidenced by their immunity against the heterologous strain is less important, although it is interesting to observe that the mice failed to develop any detectable immunity against the related strain. If the mice had been more uniformly protected against the homologous strain, the lack of immunity against the heterologous strain would be important, for Type III immunization of horses does give antibodies reactive with the Thomas strain, and Thomas immunization of rabbits usually gives antibodies reactive with typical Type III strains.

Immunity Response of Mice as Evidenced by Passive Protection Experiments.

Since active immunity is not always the same as passive immunity, it seemed important to determine whether or not the serum of the vaccinated mice could confer passive protection to other mice.

In order to eliminate the factor of differences in the individual responses of the vaccinated mice, the serum from 8 mice vaccinated with Thomas bacteria was pooled and used as the anti-Thomas immune mouse serum; and the serum from 8 mice vaccinated with the Type III bacteria was used collectively as the anti-Type III immune mouse serum. Passive protection experiments were then made by the usual procedure with doses of 1×10^{-5} and 1×10^{-6} cc. of culture, the anti-Thomas serum being tested against both Thomas and Type III bacteria and the anti-Type III serum against both Type III and Thomas bacteria.

The results of the passive protection experiments were in all respects analogous to those obtained in the previously described active immunity tests. The factor of differences in the individual responses of the vaccinated mice was eliminated by pooling the immune serum. The anti-Type III immune mouse serum gave passive protection against Type III alone and not against the related Thomas bacteria; and the anti-Thomas immune serum gave protection against the Thomas bacteria and not against the typical Type III strain. The immune mouse sera, however, are scarcely comparable in homologous potency to the anti-Type III horse serum or the anti-Thomas rabbit serum; and hence their lack of heterologous potency is of less importance, for it is common experience that an antiserum of a slight degree of homologous potency is more usually of a higher degree of specificity.

Tests with a Recently Isolated Strain of Typical Type III Pneumococcus of Different Origin from the Type III Strain Employed in Vaccination of the Mice.

In the preceding experiments, the mice were tested against the same strain of typical Type III pneumococci as that employed in the vaccination. In the previous investigation (1), the immunological relationships evidenced in rabbit and horse antisera between this strain of Type III pneumococci and the Thomas strain were proved to hold true for three other strains of Type III pneumococci which had been recently isolated in Nashville. While there was this evidence in favor of the immunological homogeneity of typical Type III strains, it seemed important to repeat the experiments with a Type III strain known to be of different origin from the Rockefeller strain with which the mice had been vaccinated. A strain of Type III pneumococci which had been isolated about 10 days previously from a patient in the Vanderbilt University Hospital was utilized for this purpose in active immunity tests against mice vaccinated with the Rockefeller Type III strain and against mice vaccinated with the Thomas strain. Passive protection tests were also made as described previously with both anti-Type III and anti-Thomas immune mouse serum.

The results were exactly the same as those reported for the tests with the strain which had been used in vaccination. The mice vac-

cinated with the Rockefeller Type III strain were protected and the mice vaccinated with the Thomas strain were not protected against the Nashville Type III strain. Similarly, the immune serum from mice vaccinated with the Rockefeller Type III strain did give, and the serum from the mice vaccinated with the Thomas strain did not give passive protection to other mice against the Nashville Type III strain.

These tests were made against only the one foreign strain and are not presented as an argument for the homogeneity of the Type III group of pneumococci. But the identical results obtained in these experiments together with those in the preceding investigation, prove that the failure of reciprocal protection of mice against Type III pneumococci by active or passive immunization with the Thomas strain, and the failure of reciprocal protection of mice against the Thomas strain by active or passive immunization with the Rockefeller typical Type III strain, are not phenomena dependent upon the use of precisely the same Type III strain in the immunization and subsequent immunity tests.

Virulence of the Thomas Strain and of the Typical Type III Strain for Rabbits.

In view of the marked differences between the relative immunity responses of mice and those of rabbits to immunization with typical Type III strains and the Thomas strain, it is important to record the tests of the virulence of these strains for the two species of animals. Both the Thomas strain and the typical Type III strain (A 66, Hospital of The Rockefeller Institute) were highly virulent for mice, killing them regularly when doses of 1×10^{-8} cc. of broth culture were injected; but it has been shown by Tillett (5) that the virulence of Type III pneumococci for mice is by no means an index of the virulence of the same strains for rabbits.

Because of the probably marked differences in the natural immunity of different individual rabbits which has been suggested by Tillett, four rabbits were tested with each strain. The individual doses were 1 cc. intravenously, 5 cc. intravenously, 5 cc. intraperitoneally and 10 cc. intraperitoneally. None of the rabbits died even from the largest doses of the typical Type III strain. Two of the rabbits (those injected with 5 cc. and with 10 cc. intraperitoneally) were killed by the Thomas strain. The cultures injected had not been passed through rabbits.

From these results, it is evident that, although neither strain is highly virulent (at least without exaltation of potential virulence by rabbit passage), the Thomas strain is more virulent for rabbits than is the typical Type III strain.

DISCUSSION.

The preceding experiments have dealt with the relative effectiveness of the responses of mice and of rabbits to immunization with a typical Type III strain in comparison with their response to immunization with the Thomas strain which is related to but not identical with Type III pneumococcus.

In regard to the response of rabbits, the apparent relationship between the antigenic effectiveness of the two kinds of pneumococci was the same as that already described in the preceding paper; but the results of the present study are important in that the conditions of immunization and the dosage were kept constant in the animals injected with the two strains. From the standpoint of the development of homologous, specific antibodies, the Thomas strain was incomparably more effective; the anti-Thomas rabbit sera having, as usual, a high degree of anti-Thomas potency; and the anti-Type III rabbit sera being, as is frequently the case, devoid of anti-Type III potency. The greater effectiveness in rabbits of the Thomas antigen was also true from the standpoint of anti-Type III potency; and the results in this investigation, as in the preceding one, furnish evidence that rabbits can produce antibodies specifically reactive with typical Type III pneumococci, more readily when injected with the Thomas strain than when injected with typical Type III pneumococci themselves.

With mice, the results were quite different. In the experiments with these animals, it was found that vaccination with typical Type III pneumococci elicited somewhat better immunity responses,—as indicated by the higher percentage of individuals which were protected,—than did vaccination with the Thomas strain. The immunity in both instances was limited to homologous protection, for both the Thomas strain (which in rabbits invoked an anti-Type III response) and the typical Type III strain (which in horses invokes an anti-Thomas response) failed to invoke an immunity against the related but different kind of Pneumococcus. From the standpoint of the

active immunity exemplified by homologous protection, the typical Type III strain proved not only to be more effective antigenically than was the Thomas strain, but also, in comparison with the results of a previous study (4), it appeared to be a more effective antigen in mice than are Type II pneumococci,—although the latter, like the Thomas pneumococci, almost invariably give better immunity responses in rabbits.

Thus, the results show that the relative antigenic effectiveness of these two kinds of related pneumococci, is different in different animals; in rabbits, the Thomas strain is more effective than typical Type III; in mice, the typical Type III is better than Thomas. These relationships furnish an example of the influence of the species of animal upon the apparent effectiveness of bacterial antigens, for while the potential antigenicity is resident in the chemical structure of the antigen, its actual effectiveness in practice is determined by the individual response of the particular animal. This example of the marked differences in the selective effectiveness of the two different antigens is the more interesting in view of the close serological relationship of the two kinds of pneumococci utilized as antigens.

SUMMARY.

The paper reports an experimental comparison of the antibody responses of mice and of rabbits to immunization with typical Type III pneumococci and a strain related to Type III. The results as a whole show that the relative antigenic effectiveness of these two kinds of related pneumococci, is different in different animals. In rabbits the strain related to but not identical with Type III elicits the better response; in mice, the typical Type III strain is more effective. These relationships furnish an example of the influence of the species of animal upon the effectiveness of even closely related bacterial antigens.

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