STUDIES ON IMMUNITY TO PNEUMOCOCCUS MUCOSUS (TYPE III).

I. Antibody Response of Rabbits Immunized with Type III Pneumococcus.

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The absence of demonstrable type specific antibodies in the serum of rabbits immunized with Type III pneumococcus has been the common experience of investigators working with this organism (1–4). Yoshioka (5) obtained type specific agglutinins in the serum of immunized rabbits, but with irregularity. He attributed his positive results to the use of one strain of Type III, since, with two other strains, no specific antibody production was elicited. Immunization of sheep and horses with Type III pneumococcus has resulted in the development of type specific agglutinins in the sera of these animals (6), but the titre has been low and passive protection slight.

This experience with *Pneumococcus mucosus* is but a confirmation of the earlier observations (7) that the serum of animals immunized with certain of the encapsulated organisms fails to show agglutinins. This failure to demonstrate antibodies in serum under these conditions has been usually attributed to the inagglutinability of organisms possessing a mucoid capsule or to the absence of agglutinins in the test serum. Porges (8) found that if encapsulated Friedländer bacilli were first subjected to a process of decapsulation, they then became agglutinable in immune serum. Hanes (1) reported that Type III pneumococci were agglutinated in their homologous immune serum following decapsulation of the organisms by means of the Porges method.

In the course of a series of investigations on immunity to Type III pneumococcus, the response of rabbits to immunization with this organism was investigated.

EXPERIMENTAL.

Antigen.—Three different strains of Type III pneumococcus were employed. These strains were derived from the blood of patients suffering from lobar pneumonia and possessed all the characteristics of Type III. They were Gram-positive diplococci; they showed capsule formation when suitably stained; they grew on blood agar plates with the production of large mucoid colonies; they were bile-soluble; they agglutinated promptly in Type III antipneumococcus horse serum; they were virulent for mice in 0.0000001 cc. doses. Both living organisms and vaccine were used for injection. A 16 hour plain broth culture was used for the living organisms. The vaccine was made by centrifuging such a culture, resuspending the organisms in physiological salt solution, and heating at 56° for half an hour.

Methods of Immunization.—Three methods of immunization were employed. (1) The intravenous injection of vaccine according to the method described by Cole and Moore (9). This method consists in alternating for 6 weeks a week of daily injections of 1 cc. of vaccine with a week of rest. (2) Following a week of intravenous vaccine injections of 1 cc. each day, living organisms were injected at 3 to 4 day intervals in increasing amounts from 0.1 to 10 cc. over a period of 4 weeks. (3) Living organisms were injected intravenously from the beginning of immunization; 1 cc. was given daily for 6 days followed by increasing doses from 3 to 10 cc. at 3 to 4 day intervals over a period of 4 weeks.

All animals were bled 10 to 12 days after the last immunizing dose.

Agglutinin Reaction.—0.5 cc. of a saline suspension of heat-killed organisms was added to an equal volume of the serum dilutions. In the tests with type specific pneumococci, a positive reaction, if present, usually appeared immediately and the final reading was made after the tubes had remained 2 hours in the water bath at 37°C. When decapsulated Type III pneumococci or R strains of pneumococci were used, the reading was made after the tubes had remained 2 hours in the water bath at 37°C. and overnight in the ice box.

Precipitin Reaction. Nucleoprotein.—The nucleoprotein solutions of Pnuemococcus used as precipitinogen in the tests were made by the method described by Avery and Morgan (10). They contained approximately 3 mg. of N per cc. 0.2 cc. of the test serum diluted to 0.5 cc. by the addition of physiological salt solution was added to 0.5 cc. of the nucleoprotein solution. Readings were made after 2 hours in the water bath at 37°C. and overnight in the ice box.

Soluble Specific Substance.—This substance, representing the N-free carbohydrate derived in a purified state from Type III pneumococci according to the method described by Heidelberger and Avery (11), was used in dilutions of 1–10,000, 1–20,000, 1–40,000, and 1–100,000. Dilutions of this degree were employed in order to escape the inhibition zone experienced (12) when higher concentrations are tested. 0.2 cc. of serum diluted to 0.5 cc. by the addition of physiological salt solution was added to 0.5 cc. of the different dilutions of the soluble specific substance. The precipitin reaction is usually immediate; however, the final reading was made after 2 hours in the water bath at 37°C.

Passive Protection Tests.—These tests were carried out in mice by the simultaneous injection intraperitoneally of 0.2 cc. of serum and culture dilution, according to the method previously described (13).

The results obtained in the experiments to be reported involve the activities of two antigen-antibody systems. In order to avoid confusion as to nomenclature, certain terms used throughout the body of the report will be defined. It has been shown by Avery and Heidelberger (14), that pneumococci are capable of stimulating the production of two distinct antibodies, depending in a large measure on the nature of the bacterial substances used as antigens. An intact pneumococcal cell possessing the soluble specific substance, when employed antigenically, stimulates the production of antibodies which are reactive with the homologous soluble specific substance. If the soluble specific substance is present in the cells used in the agglutination test, type specific agglutination occurs; if the soluble specific substance is present in solution, precipitation of this substance occurs. The antibody, which is capable of agglutinating the type specific Pneumococcus and of precipitating the type specific soluble substance will be designated as *anti-S*. The evidence of all previous work indicates that the efficacy of serum in the passive protection of mice against pneumococcus infection depends on the presence in the serum of anti-S.

The second antigen-antibody system involves the nucleoprotein fraction of the cell and the antibody response elicited by it. Pneumococcus nucleoprotein, in contrast to the soluble specific substances, is without type specificity. The degraded R strains of pneumococci also are without type specificity. Therefore the use, antigenically, of either nucleoprotein substance or R strains results in the production of non-type specific antibodies, which precipitate the nucleoprotein derived from any Pneumococcus and agglutinate all R strains. These antibodies will be designated as *anti-P*.

I. Agglutinins.

Absence of Agglutinins for the Encapsulated Type III Pneumococcus in the Sera of Animals Immunized with Type III Pneumococcus.

Twenty-eight rabbits were used in these immunization experiments. Twenty received vaccine alone; six received vaccine and living organ-

isms; two received living organisms alone. Their sera were then tested for the presence of agglutinins against the encapsulated Type III pneumococcus. Out of the twenty-eight rabbits, the sera of twenty-four failed to show type specific antibodies (anti-S). In the four positive sera type specific antibodies (anti-S) were present in low titre and in only one was the agglutinin reaction positive in a dilution of 1 to 20 (Table I). Such results are confirmatory of the experience of others in working with Type III and are in striking contrast to the results obtained when rabbits are immunized with Pneumococcus Type I or Type II, whereby type specific antibodies are readily demonstrable in the immune sera.

Presence of Agglutinins for R Strains of Pneumococcus in the Sera of Rabbits Immunized with Type III Pneumococcus.

In demonstrating the twofold antigenicity of pneumococcus substances, Avery and Heidelberger (14) showed that when intact pneumococcal cells are used as antigen the resultant antibodies consist chiefly of type specific agglutinins (anti-S) for the homologous organism; when ruptured cells or solutions of pneumococci are used as antigen the antibody response is predominantly of the anti-P character. Since the sera of rabbits immunized with Type III pneumococcus fail, in most instances, to show anti-S antibodies, and in view of the dual antigenic nature of pneumococci, these immune sera were tested for the presence of anti-P antibodies. Anti-P antibodies are reactive against the degraded R strains of Pneumococcus. As has been brought out by Reimann (15, 16) R strains are avirulent, non-encapsulated, and non-type specific. They may be derived from any of the fixed types of Pneumococcus and are agglutinated by any anti-P serum. Therefore, R strains of pneumococci are adequate in testing for the presence of anti-P antibodies. The results recorded in Table I were obtained by testing the sera of rabbits, immunized with Type III pneumococcus, for the presence of anti-P antibodies, as evidenced by the agglutination of an R strain. The sera of twenty-seven of twenty-eight rabbits possessed anti-P antibodies.

The results of this experiment indicate that rabbits immunized with Type III pneumococcus—even though this organism is one of the fixed types—react in the majority of instances by the production, not

of the type specific anti-S antibody, but of the anti-P antibody. The immunological response is identical with that obtained following immunization with R strains or solutions of pneumococci. The inference is, then, that Type III pneumococci are so altered, following introduction into the animal body, that the type specific antigen is made ineffectual and the nucleoprotein is liberated to stimulate the production of anti-P antibodies.

Agglutination of Decapsulated Pneumococci in the Sera of Rabbits Immunized with: (1) Type III Pneumococcus, (2) R Strains of Pneumococcus, (3) Nucleoprotein Derived from Pneumococcus.

Hanes (1), as previously stated, showed that Type III pneumococci, deprived of their capsule by means of the Porges (8) method, were agglutinated in the sera of rabbits immunized with Type III pneumococci; whereas, when the encapsulated organisms were tested in the same immune sera, no agglutination occurred. Both Porges and Hanes infer that the inagglutinability of encapsulated organisms is due, not to the absence of agglutinins in the test serum, but to the presence of the capsule around the organism. As proof of this they show that removal of the capsule is followed by agglutination.

Since it has been shown in Table I that rabbits immunized with Type III pneumococcus possess anti-P antibodies and, since the chief characteristic of anti-P antibodies is the agglutination of non-encapsulated pneumococci, an explanation of the results of Hanes is made possible. R strains of pneumococci agglutinated by Type III immune rabbit serum (Table I) are organisms which have been deprived of their capsule by cultural methods. The Type III organisms which Hanes found agglutinable were deprived of their capsule by a chemical procedure. The agglutination of both of these non-encapsulated organisms is identical (Table I) in Type III immune rabbit serum. Their immunological identity is further brought out by testing the agglutination of chemically decapsulated organisms in the sera of rabbits immunized with R strains and with pneumococcus nucleoprotein. The results (Table II) show that any anti-P serum is capable of agglutinating the chemically decapsulated Type III pneumococcus and R strains equally well. In reports on Friedländer bacilli Julianelle (17) obtained similar results; namely, that decapsulated and R

TABLE I.

Agglutination of Encapsulated Type III Pneumococcus, Decapsulated Type III Pneumococcus, and of an R Strain of Pneumococcus in the Sera of Rabbits Immunized with Type III Pneumococcus.

Rabbit No.	Agglutinins for Pneumococcus III encapsulated					Agglutinins for Pneumococcus III decapsulated					Agglutinins for R_3 strain of pneumococcus					
	Serum dilutions					Serum dilutions					Serum dilutions					
	1–2	1–10	1–20	1–40	1–80	1–10	1–20	1–40	1–80	1–160	1–2	1–10	1–20	1–40	1–80	1–160
1	−	−	−	−	−	++++	+++	+	±	−	++++	++++	+++	++	±	−
2	−	−	−	−	−	++++	++++	++	+	+	++++	++++	++	+	−	−
3	−	−	−	−	−	++++	++	+	−	−	++++	+++	++	+	−	−
4	−	−	−	−	−	++++	+++	+	±	−	++++	++++	++	+	−	−
5	−	−	−	−	−	++++	++++	+++	+	±	++++	++++	+++	++	+	±
6	−	−	−	−	−	++++	+++	+	±	−	++++	+++	+	±	−	−
7	−	−	−	−	−						+++	++	+	−	−	−
8	−	−	−	−	−						++++	++++	+++	++	+	±
9	−	−	−	−	−						++++	+++	++	+	−	−
10	−	−	−	−	−						++++	+++	++	+	±	−
11	−	−	−	−	−						+++	++	±	+	−	−
12	−	−	−	−	−						++++	+++	++	++	±	−
13	−	−	−	−	−						++++	+++	++	+	−	−
14	−	−	−	−	−						++++	++++	+++	++	+	−
15	××××	××	×	×	−						+	±	−	−	−	−
16	×××	×	−	−	−						++	++	+	−	−	−
17	××	×	−	−	−						++++	+++	+	±	−	−
18	−	−	−	−	−						++++	++++	++	++	±	−
19	−	−	−	−	−						++++	++++	+++	+	−	−
20	−	−	−	−	−						±	−	−	−	−	−
21	−	−	−	−	−						+++	+	±	−	−	−
22	−	−	−	−	−						++++	+++	+	±	−	−

23	−	−	−	−	−						++++	++++	++	+	±	−
24	−	−	−	−	−						++++	++++	+++	++	+	±
25	−	−	−	−	−						++++	+++	++	+	−	−
26	−	−	−	−	−						++++	++++	++	+	±	−
27	××××	××	×	−	−						++++	+++	+	±	−	−
28											++++	++++	++	+	±	−

Controls: 0.5 cc. organisms + 0.5 cc. of salt solution: diffuse. 0.5 cc. organisms + 0.5 cc. of normal rabbit serum: negative.

×××× indicates characteristic compact disc of type specific agglutination with clear supernatant fluid. ××× indicates compact disc with faintly cloudy supernatant fluid. ×× indicates disc which can be partially broken up with cloudy supernatant fluid. × indicates coarse granulation. $\underline{\times}$ indicates doubtful granulation. − indicates negative.

++++ indicates complete agglutination with clear supernatant fluid. +++ indicates less coarse agglutination with faintly cloudy supernatant fluid. ++ indicates agglutination with cloudy supernatant fluid. + indicates some granulation both sedimented and diffuse. ± indicates some sedimentation easily made diffuse. − indicates negative.

TABLE II.

Agglutination of Decapsulated Type III Pneumococci and of R Pneumococci in the Sera of Rabbits Immunized with Two Different R Strains of Pneumococcus and with Pneumococcus Nucleoprotein.

		Decapsulated Type III						R_2 pneumococci					
Rabbit No.	Immunized with	1–10	1–20	1–40	1–80	1–160	1–320	1–10	1–20	1–40	1–80	1–160	1–320
29	R_1 strain	++++	++++	+++	+	±	−	++++	+++	++	+	±	−
30	R_2 strain	++++	+++	+	±	−	−	++++	+++	+	−	−	−
31	Nucleo-protein	++++	+++	++	+	±	−	++++	+++	++	+	−	−

Controls: 0.5 cc. of organisms + 0.5 cc. of salt solution: diffuse. 0.5 cc. of organisms + 0.5 cc. of normal rabbit serum: some sedimentation which was made diffuse by slight tapping.

++++ indicates complete agglutination with clear supernatant fluid. +++ indicates less coarse agglutination with faintly cloudy supernatant fluid. ++ indicates agglutination with cloudy supernatant fluid. + indicates some granulation both sedimented and diffuse. ± indicates some sedimentation easily made diffuse. − indicates negative.

strains of Friedländer bacilli were identical in their agglutination reactivity in anti-P (Friedländer) serum.

II. Precipitins.

It was shown by Reimann (16) that sera which contained agglutinins (anti-P) for R strains of Pneumococcus also contained precipitins for pneumococcus nucleoprotein solutions. Therefore, since the sera of the rabbits immunized with Type III pneumococcus possessed anti-P agglutinins, they were tested for the presence of precipitins for pneumococcus nucleoprotein (Table III).

A comparison of Table III with Table I will show that all the sera which contained anti-P agglutinins also possessed anti-P precipitins. Since the nucleoprotein in this test was obtained from a Group IV pneumococcus, the possibility of a type specific reaction is excluded and the experiment further identifies the antibody present in the Type III immune sera as of an antiprotein character.

The sera of the rabbits immunized to Type III were also tested for the presence of precipitins against Type III specific soluble substance (Table III). As has been shown by Avery and Heidelberger (14), the type specificity of pneumococci depends upon the presence of this substance. They have obtained it in a highly purified state from each of the three fixed types of Pneumococcus. Immune sera which possess type specific agglutinins (anti-S) for pneumococci of a fixed type also possess precipitins for the soluble specific substance derived from that type. The results obtained with Type III immune rabbit sera used in these experiments show the concomitant occurrence of Type III specific agglutinins (anti-S) and precipitins for the Type III soluble specific substance. On the other hand, those sera which failed to show type specific agglutinins (anti-S) also failed to precipitate the soluble specific substance. This is further evidence that the lack of agglutination of the encapsulated Type III cell in homologous immune rabbit serum is due to the actual absence of the anti-S antibody in demonstrable quantity since such factors as were supposed to render the encapsulated organism inagglutinable are not present in the precipitin test.

TABLE III.

Precipitation of the Soluble Specific Substance of Type III Pneumococcus and of a Solution of Pneumococcus Nucleoprotein in the Sera of Rabbits Immunized with Type III Pneumococcus.

Rabbit No.	Precipitins for soluble specific substance of Type III pneumococcus				Precipitins for solution of pneumococcus nucleoprotein
	Dilutions of soluble specific substance				Dilution of protein
	1–10,000	1–20,000	1–40,000	1–100,000	1–300
1	–	–	–	–	+++
2	–	–	–	–	++
3	–	–	–	–	++
4	–	–	–	–	++
5	–	–	–	–	+++
6	–	–	–	–	++
7	–	–	–	–	++
8	–	–	–	–	+++
9	–	–	–	–	++
10	–	–	–	–	++
11	–	–	–	–	++
12	–	–	–	–	++
13	–	–	–	–	++
14	–	–	–	–	++
15	××××	××	×	×	+
16	××××	×	×	–	±
17	×××	×	–	–	++
18	–	–	–	–	++
19	–	–	–	–	++
20	–	–	–	–	–
21	–	–	–	–	+
22	–	–	–	–	++
23	–	–	–	–	++
24	–	–	–	–	+++
25	–	–	–	–	++
26	–	–	–	–	++
27	××××	×	×	–	++
28	–	–	–	–	+++

Controls: 0.5 cc. of various dilutions of soluble specific substance + 0.5 cc. salt solution: clear. 0.5 cc. of various dilutions of soluble specific substance +0.2 cc. of normal rabbit serum + 0.3 cc. of salt solution: clear. 0.5 cc. of pneumococcus nucleoprotein solution + 0.5 cc. of salt solution: slightly hazy

III. Passive Protection in Mice.

The sera of the twenty-four rabbits immunized with Type III pneumococcus were tested for their ability to protect mice against infection with Type III pneumococcus. The dependence of passive protection on the presence of type specific antibodies (anti-S) in the test serum is well known to investigators working in experimental pneumococcus immunity. Therefore, it is to be expected that those sera containing anti-S afford some protection and that those without it fail. The results recorded in Table IV show that protection parallels the presence of anti-S and not anti-P. Of the twenty-four sera tested, the four possessing anti-S showed protection; of the twenty possessing no demonstrable anti-S, seventeen afforded no protection, and three afforded very slight protection. The explanation of the results obtained with the latter three sera probably rests on the fact that these sera contained sufficient type specific antibodies (anti-S) to afford some protection but not sufficient to be demonstrable by test-tube agglutination. That the mouse protection test is a more delicate test for the presence of anti-S than test-tube agglutination may be brought out by the use of Type I antipneumococcus serum. This serum may be diluted to a point where no test-tube agglutination is demonstrable and yet such a dilution of serum affords some protection. A further report will be made later on the results obtained in passive protection following the use of the sera of rabbits immunized with Type III pneumococci.

An analysis of the antibodies present in the sera of twenty-eight

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0.5 cc. of pneumococcus nucleoprotein solution + 0.2 cc. of normal rabbit serum + 0.3 cc. of salt solution: slightly hazy.

×××× indicates characteristic compact disc of type specific precipitin reaction with clear supernatant fluid. ××× indicates compact disc with faintly cloudy supernatant fluid. ×× indicates smaller disc at bottom of tube with cloudy supernatant fluid. × indicates cloudy fluid. × indicates very faintly cloudy fluid. − indicates negative.

+++ indicates precipitate at bottom of tube with faintly cloudy supernatant fluid. ++ indicates slight precipitate with cloudy supernatant fluid. + indicates cloudy fluid. ± indicates very faintly cloudy fluid. − indicates negative.

rabbits immunized with Type III pneumococcus may be summarized as follows:

Agglutinins.—Twenty-four failed to show type specific agglutinins (anti-S). Four possessed type specific agglutinins (anti-S) in low titre. Twenty-seven possessed anti-P agglutinins in appreciable titre. One failed to show evidence of any antibody response.

TABLE IV.

Summary of Passive Protection in Mice by the Use of Sera of Rabbits Immunized with Type III Pneumococcus.

	Demonstrable anti-S antibodies		Demonstrable anti-P antibodies		Rabbit No.	Passive protection in mice against Type III pneumococcus infection. Dose of culture			
	Agglutinins	Precipitins	Agglutinins	Precipitins		0.001 cc.	0.0001 cc.	0.00001 cc.	0.000001 cc.
Sera of 17 rabbits	Absent	Absent	Present	Present		D.	D.	D.	D.
Sera of 3 rabbits	Absent	Absent	Present	Present	6	D.	D.	D.	S.
					11	D.	D.	D.	S.
					12	D.	D.	S.	S.
Sera of 4 rabbits	Present	Present	Present	Present	15	S.	S.	S.	S.
					16	S.	S.	S.	S.
					17	D.	S.	S.	S.
					27	D.	S.	S.	S.

D. indicates death of animal.
S. indicates survival of animal.

Precipitins.—The presence of precipitins for Type III soluble specific substance paralleled the presence of type specific agglutinins. The presence of precipitins for pneumococcus nucleoprotein paralleled the presence of anti-P agglutinins.

Passive Protection in Mice.—Four sera possessing demonstrable anti-S antibodies afforded some protection against Type III infection.

Of twenty sera without demonstrable anti-S, seventeen failed to confer any protection; three afforded minimal protection.

DISCUSSION.

The common experience that Type III pneumococci, antigenically employed, fail to stimulate the production of type specific agglutinins (anti-S) has been encountered in a great majority (85.8 per cent) of the rabbits used in the experiments here reported. It is a striking fact that such a cell, possessing a large amount of soluble specific substance, and highly virulent for mice, acts so feebly in stimulating type specific antibodies (anti-S); whereas Type I and Type II pneumococci, possessing the same qualities, are so effective in producing homologous type specific antibodies. However, the presence of agglutinins for R pneumococci and precipitins for pneumococcus nucleoprotein (anti-P antibodies) is evidence that Type III pneumococci are not without an antigenic component. The results of these experiments reveal the fact that the antibody response of rabbits to immunization with the encapsulated Type III pneumococcus is identical with the antibody response obtained by immunization with solutions of pneumococci. In both instances the production of anti-P antibodies has been stimulated. Such a result could be accomplished only by *in vivo* disruption of the pneumococcal cells. The inference, then, may be drawn that normal rabbits possess a mechanism whereby Type III pneumococci, following intravenous injection, are disintegrated in such a manner that the part of Type III antigenic complex, which stimulates type specific agglutinins (anti-S), is destroyed. The nucleoprotein fraction of the cell, however, remains capable of stimulating anti-P antibodies.

The identification of the antibody present in Type III immune rabbit serum, as being anti-P, is explanatory of Hanes' results with decapsulated Type III pneumococci. By decapsulation, Type III pneumococci are reduced to an R form, and are agglutinated by anti-P antibodies. The failure of encapsulated Type III to agglutinate is due to the actual absence of anti-S antibodies.

CONCLUSIONS.

1. Type III pneumococci fail in the majority of instances to stimulate the production of anti-S antibodies. (Type specific agglutinins, type specific precipitins, and antibodies affording type specific protection in mice.)

2. Type III pneumococci are effective in the stimulation of the production of anti-P antibodies (agglutinins for R strains of pneumococci and precipitins for pneumococcus nucleoprotein). These antibodies are ineffectual in the passive protection of mice.

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