THE ANTIGENIC PROPERTIES OF SOLUTIONS OF PNEUMOCOCCUS.

By OSWALD T. AVERY, M.D., and JAMES M. NEILL, Ph.D.

(From the Hospital of The Rockefeller Institute for Medical Research.)

(Received for publication, June 8, 1925.)

In the preceding paper (1) the immunological properties of intact pneumococci were compared with those of the isolated bacterial protein and carbohydrate. The whole cell, with antigenic functions unimpaired, gives rise to immune bodies which agglutinate pneumococci of the homologous type and precipitate the soluble specific substance, the carbohydrate, of the cell. It was shown that the bacterial protein stimulates the formation of precipitins which react with pneumococcus protein regardless of the type. This antibody to the protein does not agglutinate type-specific strains or precipitate solutions of the specific polysaccharide which distinguishes each type of Pneumococcus. The carbohydrate substance when dissociated from the bacterial cell has been found to possess no antigenic power; that is, in the free state it it is devoid of the property of inciting antibody formation. Antibodies reactive with the free carbohydrate can be produced only by immunization with intact organisms.

The subject of the present paper is the marked difference in the antigenic properties of intact pneumococci and the bacterial substances of these same organisms when in solution.

EXPERIMENTAL.

In the course of our previous studies on cellular oxidations by pneumococci, cell solutions or extracts of these organisms were prepared by a special method (2). These solutions, freed from all formed and living elements by Berkefeld filtration, were found to exhibit many of the physiological activities of the living cell. In addition to the active intracellular agents which function in cellular processes, they contain the constituents of the cell upon which its

immunological properties depend; namely, the pneumococcus carbohydrate and protein. Such cell solutions were employed, therefore, in the present study in order to compare the antigenic properties of dissolved pneumococci with those of the bacterial cells as such.

Methods.—Cell solutions of pneumococci were prepared by suspending the centrifuged bacteria from young broth cultures in small amounts of salt solution or culture fluid so that 1 cc. of the suspension contained the organisms from 50 cc. of the original broth culture. These concentrated cell suspensions were frozen and thawed repeatedly; the residual material was removed by centrifugation, and the supernatant fluid was passed through a Berkefeld filter. These extracts contain, therefore, no formed cell elements. They were proven sterile by cultural and mouse tests.

For purposes of comparative study, heat-killed suspensions of pneumococci were employed for immunization. The bacteria from 8 hour plain broth cultures were centrifuged and resuspended in salt solution in a volume equal to one-tenth that of the original culture. The bacterial suspensions were immediately heated in a water bath at 56°C. for 30 minutes. Young, actively growing cultures were used, and the bacterial suspensions were heated at once in order to minimize the amount of autolysis and cell solution which invariably result if such precautions are not observed. The reason for emphasizing these points will be apparent in the following experiments.

Immunization.—The sterile cell solutions were injected into rabbits intravenously on 6 consecutive days, followed by a free interval of 1 week. Four courses of injections were given, the doses gradually increasing from 0.1 cc. until a maximum of 2 cc. was administered daily in the last series of injections.

The suspensions of heat-killed bacteria were injected intravenously into rabbits in six daily doses of 0.1 cc. each, this amount of concentrated vaccine being suitably diluted to a volume of 1 cc. with salt solution at the time of injection. Four iuch courses of injections at weekly intervals were given, the animals receiving sn all the equivalent of bacteria from 24 cc. of culture. The rabbits were bled on the 10th day after the last injection.

Tests.—In all precipitin reactions 0.2 cc. of immune serum, diluted to 0.5 cc. volume with salt solution, was added to an equal volume of graded dilutions of the substances to be tested, either the isolated carbohydrate or the protein fraction of the cell, or solutions of both substances as they occur in extracts of the original cells. Mixtures of the reacting substances were incubated for 2 hours at 37°C. The technique employed in agglutination and protection tests was the same as that described in detail in earlier papers.

In the following experiments the immune sera of rabbits injected with the cellular substances of Pneumococcus in solution and in the form of intact organisms were tested for the presence of (1) precipitins, (2) agglutinins, and (3) protective antibodies.

1. Precipitins for the Carbohydrate of Pneumococcus.—In order to determine whether or not rabbits immunized to solutions of pneumococci develop specific antibodies reactive with the soluble specific substance (carbohydrate), the sera of these animals were tested by the precipitin reaction against dilutions of this substance isolated in purified form from the three fixed types of pneumococci.

Repeated experiments have revealed the interesting fact that the sera of rabbits immunized with solutions of the cellular substances of pneumococci do not contain any demonstrable precipitins for the specific carbohydrate of the cell. A typical protocol is presented in

TABLE I.

Precipitins for the Type-Specific Carbohydrate of Pneumococcus in the Sera of Rabbits Immunized with Intact Organisms and with Solutions of the Cellular Substances.

Immune sera.	Specific carbohydrate isolated from Pneumococcus.						
	Type I.		Type II.			Type III.	
	1:1000	1:4000	1:4000	1:80,000	1:2,000,000	1:4000	1:80,000
Rabbit 1 (solutions of Pneumococcus Type II)	−	−	−	−	−	−	−
Rabbit 2 (intact Type II pneumococci)	−	−	+++	++++	++	−	−

++, turbidity; +++, turbidity and flocculation; ++++, rapid flocculation with compact sedimentation; −, clear, no reaction.

Table I. The presence of this carbohydrate antibody in bacterial immune sera, and its complete absence from the sera of animals immunized with solutions of the same cellular substances, indicate that the morphological integrity of the cell is essential for the development of its full antigenic power.

No matter whether dissolution of the bacterial cells is effected by the freezing and thawing process or by the lytic action of bile, the resulting solution, although containing both the carbohydrate and the protein substances of the whole bacterium, fails to stimulate the formation of the type-specific antibodies which react with the homologous

carbohydrate and which are always dominant in sera prepared by the injection of intact cells. The fact that the polysaccharide in the formed cell is specifically antigenic and that the same substance in cell solutions is non-antigenic forces the conclusion that the carbohydrate exists in the cell not merely as a free polysaccharide, but in combination with some other substance which confers antigenic properties upon it. With rupture of the cell and the passing into solution of its constituents this linkage is broken, and the free carbohydrate fraction, although still retaining the property of specific union with its type antibody, is itself no longer capable of inciting the formation of this same immune body. In summary, then, cell disintegration is accompanied by dissociation of the dominant antigenic complex, and the cell constituents thus dissociated exhibit immunological properties quite different from those characteristic of the formed cell.

2. Precipitins for the Protein of Pneumococcus.—The preceding experiments have shown that sera obtained by injection of solutions of the cellular substances contain no precipitins for the specific carbohydrate of the homologous type of Pneumococcus. In the following experiments the sera were tested for precipitins against the so called nucleoprotein isolated from pneumococci of homologous and heterologous types, and for their capacity to precipitate bacterial solutions containing, in addition to the carbohydrate, the cell protein unchanged by chemical treatment.

"Nucleoprotein," precipitated from solution by dilute acetic acid in the cold, was prepared from dissolved pneumococci of Types I and II and Group IV. This material, while subject to the changes incident to denaturation and representing a mixture of mucoid and other proteins, is referred to as nucleoprotein not to imply its exact nature, but to distinguish it from the protein-free carbohydrate fraction of the cell. Both of these cellular substances were separately tested in varying concentration against a constant amount of immune serum. The protein solutions were used in dilution of 1:400 and 1:4000, and the carbohydrate was employed in optimum concentration to avoid prozone inhibition.

Many experiments similar to that outlined in Table II have demonstrated the fact that animals immunized with dissolved pneumococci containing both the carbohydrate and protein of the cell in solution develop in their serum an antibody which is reactive with only one of

these substances; namely, the protein. Furthermore, whether produced as in the present instance by immunization with cell solutions or by the injection of the isolated protein alone (1), antiprotein sera react with pneumococcus protein regardless of its type derivation. Equally striking is the fact that although the material used for injection includes both the bacterial carbohydrate and protein, the sera thus developed are wholly devoid of any antibody reacting with the carbohydrate or type-specific substance of the cell.

Solutions of dissolved pneumococci, so far as their power to stimulate antibodies is concerned, behave precisely then as do solutions of pneumococcus protein alone. Although these solutions contain the

TABLE II.

Precipitins for the Protein and Carbohydrate Constituents of Pneumococcus Following Immunization with Cell Solutions of Type II Pneumococci.

Immune sera.	Nucleoprotein from Pneumococcus.				Carbohydrate from Pneumococcus.		
	Type I.	Type II.	Group IV.		Type I.	Type II.	Type III.
	Strain N.	Strain L.	Strain F.	Strain S.	Strain N.	Strain L.	Strain A.
Rabbit 1 (solutions of Pneumococcus Type II)	++	++	++	++	−	−	−
Normal rabbit	−	−	−	−	−	−	−

++, marked precipitation; −, no reaction.

two immunologically important constituents of Pneumococcus, the free carbohydrate when liberated from the cell, like the chemically purified polysaccharide itself, possesses no antigenic power. This fact is the more interesting since the carbohydrate in these cell solutions has been subjected to no chemical treatment such as is used in the preparation of the purified substance. When present in its free, dissociated state the carbohydrate neither functions as antigen nor participates in the protein precipitin reaction. That it does not function as antigen is shown by the fact that these sera are wholly devoid of the power of reacting with the type-specific carbohydrate (see Table II). That it does not participate in the protein reaction is proved by the fact that removal of the protein from cell solutions by

specific absorption leaves the carbohydrate free in the supernatant undiminished in titer.

Since antisera prepared by immunization with the cellular substances in soluble form never contain antibodies for the specific carbohydrate, while sera obtained by the injection of intact bacteria always show the presence of this antibody, it at once becomes obvious that the nature of the immune response is dependent upon the character of the material used for immunization. Because of the readiness with which Pneumococcus undergoes autolysis, suspensions of originally intact cells almost invariably contain not only formed elements but also more or less of the dissociated cell constituents in solution. Under these circumstances, antibacterial sera may and as a rule do exhibit not only the dominant type-specific carbohydrate-reacting antibody, but also in minor degree the protein antibody. The presence of this latter antibody is an expression of the difference in the antigenic properties of the same substances as they exist in the whole, complete cell, and in the dissociated state following cell disintegration. This fact is brought out in Table III, in which the serum of a rabbit immunized with suspensions of originally intact pneumococci is shown to exhibit slight reactions with the common protein in the cell solutions of heterologous types.

Table III also emphasizes the fact that immunization with cell solutions of pneumococci of one type evokes antibodies which precipitate solutions of organisms of all other types. Since immune sera prepared in this manner have been found to be reactive only with the protein and to be wholly devoid of the property of interacting with the carbohydrate of the cell, it is evident that this precipitating action is due to the presence of an antibody reactive with the protein common to all pneumococci.

For reasons already defined the serum of an animal immunized with suspensions of pneumococci may show in addition to the distinctive type-specific carbohydrate reaction a slight antiprotein reaction when tested against the protein-containing solutions of other types. The presence of a certain amount of this protein antibody in antibacterial sera, in the preparation of which supposedly only intact cells have been used, is to be expected in view of the readiness with which pneumococci undergo dissolution in the test-tube and probably also in the animal body.

The facts to be emphasized here are that when filtered solutions or extracts of bacterial substances free from all formed elements are used in immunization, there results no antibody which is reactive with the specific carbohydrate of the cell; secondly, that this type of antibody, as far as our experimental knowledge goes, is produced only by immunization with intact cells.

3. Agglutinins.—In comparing the differences in agglutinin response to immunization with pneumococci in the dissolved and formed

TABLE III.

Comparison of the Common Protein and Type-Specific Carbohydrate Precipitins Present in Immune Sera of Rabbits Immunized with Cell Suspensions and Cell Solutions of Pneumococcus Type II.

Sera of rabbits immunized with	Cell solution of pneumococci containing protein and carbohydrate of								
	Type I.			Type II.			Type III.		
	1:2	1:20	1:200	1:2	1:20	1:200	1:2	1:20	1:200
Solutions of Type II pneumococci.	++++	+++	+	++++	+++	+	++++	+++	+
Intact Type II pneumococci.....	+	±	−	xxxx	xxxx	xxxx	+	±	−

Protein reactions: ±, faint cloud delayed; +, turbidity, no precipitate; +++, turbidity and granular precipitation; ++++, marked precipitation.

Carbohydrate reaction: xxxx, immediate flocculation settling as compact disc.

The protein-antiprotein reaction varies from a cloudiness to a finely granular precipitate which is easily broken up on shaking the tube; the anti-*S* or carbohydrate reaction is characterized by a rapidly appearing, coarser flocculation which settles to the bottom of the tube as a firm, compact disc not easily broken up on agitation.

state, the sera of rabbits immunized against single strains of different types were studied. Since the nature of the reactions was identical in all cases, a type protocol of an individual serum is given in Table IV.

Cole (3) in 1914 observed that sera obtained by the immunization with bile solutions of pneumococci contain no agglutinins for the homologous type of organism. This observation is confirmed in the present study, in which the absence of specific agglutinins in the sera of rabbits treated with cell solutions has been constantly noted.

Just as these bacterial solutions, when used as antigen, fail to stimulate the formation of precipitins for the specific, homologous carbohydrate of the cell, so do they lack the property of inciting specific agglutinins for the intact organisms. Since in the case of Pneumococcus, agglutination is considered to be the result of an interaction at the periphery of the cell between the type-specific carbohydrate and its homologous antibody, the same reasons which account for the absence of the carbohydrate precipitins will explain the absence of specific agglutinins.

4. Protective Antibodies.—Rabbits were immunized with heat-killed pneumococci by the method described by Cole and Moore (4). The sera of animals which received relatively small amounts of intact bacteria afforded specific protection against 100,000 times the

TABLE IV.

Agglutinins in Rabbit Serum Following Immunization with Cell Solutions and Cell Suspensions of Pneumococcus Type II.

Serum of rabbits immunized with	Saline suspensions of pneumococci.*		
	Type I.	Type II.	Type III.
Solutions of Pneumococcus Type II	−	−	−
Intact Type II pneumococci	−	++++	−
Normal rabbit	−	−	−

−, no agglutination; ++++, complete agglutination.

* The bacterial suspensions were heated for 40 minutes at 56°C. to prevent autolysis of the cell.

dose of virulent organisms which was invariably fatal for control mice. On the other hand, the sera of rabbits injected with concentrated solutions of the dissolved bacteria were without protective value. Sera of this type, as pointed out in connection with the preceding experiments, contain antibodies reactive only with pneumococcus protein, and possess neither agglutinins for the formed cells nor precipitins for the soluble specific carbohydrate derived from them. The principles underlying the protective mechanism of anti-pneumococcus serum are not as yet sufficiently understood to relate this phenomenon to the presence or absence of any particular antibody. The data presented indicate clearly that the dissociated substances of dissolved pneumococci are not capable of eliciting the full protective

response stimulated by the bacterial cell as a whole. Just as antibodies reactive with the bacterial cell *in vitro* are evoked only when intact organisms are used as antigen, so antibodies conferring passive protection against pneumococcus infection are stimulated most readily and most effectively by immunization with the whole cell.

DISCUSSION.

It is not the purpose of the present discussion to attempt or suggest an interpretation of the experimental data in terms of their more general immunological significance. But for pneumococci, at least, they serve to point out striking differences in the antigenic properties of the bacterial substances as they exist in the cell itself and as they occur in solutions of the dissolved organisms. This difference consists in the loss of a specific antigenic function which the cell suffers whenever dissolution occurs. The function lost is the capacity to stimulate the formation of the type-specific antibodies which characterize the immune response to the uninjured bacterial cell. Antibacterial sera containing these antibodies specifically agglutinate pneumococci of the homologous type and precipitate the free carbohydrate derived from bacteria of the corresponding type. On the other hand, solutions of the same organisms containing both the carbohydrate and protein of the original cell, but free of all formed elements, fail to induce antibodies having either of these specific properties. Yet solutions of dissolved bacteria, although lacking the complete antigen of the whole cell, are not without antigenic properties. By reason of the protein they contain, these solutions induce antibodies which react with pneumococcus protein, and by virtue of the common character of this constituent in pneumococci, sera containing the protein antibody cross-react with protein from all types of pneumococci. The dominant antigenic character of the intact cell is its capacity to evoke the type-specific carbohydrate-reacting antibody, while the same cells in solution yield only the protein antibody of the species. This protein-antiprotein reaction is not limited by type differentiation, and, as Lancefield (5) has recently shown, extends to the more or less closely related cocci. Antiprotein sera obtained by immunization with dissolved pneumococci are similar in

their immune reactions to sera produced by injection of the isolated protein alone; that is, they react with pneumococcus protein regardless of its type derivation and they fail to react with the free carbohydrate or with the intact cells of the homologous type.

Previous studies from this laboratory (6) have shown that it is the carbohydrate constituent, the so called soluble specific substance of Pneumococcus, which endows the bacterial cell with type specificity. The work of Zinsser, Mueller, and their associates (7) indicates quite clearly that similar relationships hold for a number of other bacteria and yeast. Since the carbohydrate of Pneumococcus when dissociated from the cell, although still retaining its capacity to unite with antibody, loses its antigenic power, one may assume that in the intact organism this constituent exists not only as free carbohydrate, but also in combination with some other substance which confers upon it true antigenicity. What the nature of this antigenic union is, whether it is one between the carbohydrate and protein of the cell or not, is as yet undetermined. The evidence presented in the present paper indicates, however, that when the cell undergoes dissolution the specific antigenic complex is dissociated, and that the resulting solution, containing both the cell protein and carbohydrate in the free form, exhibits only those antigenic properties due to the common protein. It is not surprising, therefore, that in antipneumococcus sera there is commonly present not only the dominant type-specific antibody, but to a greater or less extent the protein antibody of the species. The presence of the latter antibody in any given lot of serum is, upon the bases of this analysis, referable to the amount of cell dissolution which has taken place more especially before and possibly to some extent after injection of the organism into the animal body.

CONCLUSIONS.

1. Intact pneumococci, possessing specific antigenic powers unimpaired by cultural or other procedures, give rise to agglutinins for organisms of the homologous type and to precipitins for the type-specific carbohydrate derived from them.

2. Solutions of pneumococci free of all formed elements, but containing the carbohydrate and protein of the original cell, fail to stimu-

late the formation of type-specific antibodies. Sera prepared in this manner do not react with the carbohydrate constituent of the cell and do not agglutinate organisms of the homologous type. The loss of this antigenic function is related to changes incurred during dissolution of the bacterial cell.

3. Solutions of the cellular substances of Pneumococcus, although lacking the specific antigen of the whole cell, induce the formation of antibodies reactive with pneumococcus protein regardless of the type from which the latter is derived.

BIBLIOGRAPHY.

1. Avery, O. T., and Morgan, H. J., *J. Exp. Med.*, 1925, xlii, 347.
2. Avery, O. T., and Neill, J. M., *J. Exp. Med.*, 1924, xxxix, 347, 357, 543, 745.
3. Cole, R., *J. Exp. Med.*, 1914, xx, 346.
4. Cole, R., and Moore, H. F., *J. Exp. Med.*, 1917, xxvi, 537.
5. Lancefield, R. C., *Proc. Soc. Exp. Biol. and Med.*, 1924–25, xxii, 109.
6. Dochez, A. R., and Avery, O. T., *J. Exp. Med.*, 1917, xxvi, 477. Heidelberger, M., and Avery, O. T., *J. Exp. Med.*, 1923, xxxviii, 73. Avery, O. T., and Heidelberger, M., *J. Exp. Med.*, 1923, xxxviii, 81. Heidelberger, M., and Avery, O. T., *J. Exp. Med.*, 1924, xl, 301.
7. Zinsser, H., and Parker, J. T., *J. Exp. Med.*, 1923, xxxvii, 275. Mueller, J. H., and Tomcsik, J., *J. Exp. Med.*, 1924, xl, 343. Zinsser, H., and Mueller, J. H., *J. Exp. Med.*, 1925, xli, 159. Mueller, J. H., *Proc. Soc. Exp. Biol. and Med.*, 1924–25, xxii, 209. Mueller, J. H., Smith, D. E., and Litarczek, S., *Proc. Soc. Exp. Biol. and Med.*, 1924–25, xxii, 373.