IMMUNOLOGICAL PROPERTIES OF A TYPICAL (S-PRODUCING) AND A DEGRADED (NON-S-PRODUCING) STRAIN OF TYPE II PNEUMOCOCCUS WITH SPECIAL REFERENCE TO PROTECTIVE ANTIBODIES.

BY EMIDIO L. GASPARI,* WILLIAM L. FLEMING, AND JAMES M. NEILL, PH.D.

(From the Department of Bacteriology and Immunology, Vanderbilt University Medical School, Nashville.)

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INTRODUCTION.

The advance in the knowledge of the chemical nature of the carbohydrate substance (1–3) of Pneumococcus makes it desirable to obtain further evidence of its biological significance. Facts of interest in this connection have been furnished by the reports (4) of the changes in immunological properties exhibited by pneumococci which have lost the specialized cellular function of elaborating the soluble specific substance (carbohydrate). Reimann's (5) recent paper adds value to the earlier work (4), since his results indicate that the immunological differences accompanying the loss of the S-producing function can be explained upon the basis of Avery and Heidelberger's interpretation of the immunological relations of pneumococcus cell constituents.

It is the object of the present paper to present evidence of similar nature, but with special emphasis upon the immunological reactions involved in the protection of mice against virulent Type II pneumococci. The study consisted of a comparison of the immunological properties of two strains of pneumococci: a type-specific strain in which the S-producing function of the cell was highly developed; and a non-type-specific strain in which the S-producing function was completely lost. The comparison of the two strains from the stand-

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point of antipneumococcus protection of mice was investigated by active and passive immunity tests, and by the absorption of antipneumococcus serum with whole bacterial cells and with culture filtrates.

EXPERIMENTAL.

Strains of Pneumococci.—Two strains of pneumococci were employed in the investigation: (1) an S-producing[1] (Type II) strain; (2) a non-S-producing[1] strain derived by the "spontaneous degradation" of the first strain. *S-Producing Strain.*—Strain D_{39}, Type II (Hospital of The Rockefeller Institute) was used as the S-producing or type-specific strain. It was highly virulent, its lethal dose for mice never being greater than one-millionth cc. of culture. *Non-S-Producing Strain.*—The non-S-producing or non-type-specific strain was derived from the above type-specific strain by repeated transfers in broth made at infrequent intervals. The culture finally used had passed through 50 successive transfers in broth at intervals of 2 to 3 days; it had completely lost its S-producing function, and was no longer pathogenic for mice.

Serological Reactions of the S-Producing and Non-S-Producing Strains of Pneumococci.

In type-specific antipneumococcus serum, the S-producing strain exhibited the usual serological reactions; the bacteria themselves were agglutinated and the carbohydrate or S substance in culture filtrates was precipitated. In contrast, the bacterial cells of the non-S-producing strain were not agglutinated, nor were the supernatants of its broth cultures precipitated. The results obtained in species-specific antipneumococcus protein serum were quite different. The S-producing strain was not specifically agglutinated, nor was the carbohydrate substance in its filtrates precipitated. The non-S-producing strain, on the other hand, was agglutinated by the serum which contained the species-specific antiprotein antibodies.

The differences between the serological reactions of the S-producing and non-S-producing strains are essentially the same as those which have been reported by Reimann. The differences in agglutinability can be explained upon the basis of Avery and Heidelberger's schematic interpretation of the structure of the pneumococcus cell. The type-specific agglutination of the S-producing strain may be

[1] The term "S-producing" strain as employed throughout this paper refers to the original, virulent, highly type-specific strain which possesses the specialized cellular function of elaborating the immunologically reactive carbohydrate substance. The same properties are exhibited by Reimann's "S" strains.

The term "non-S-producing" strain refers to the avirulent, non-type-specific strain which has lost the property of producing the soluble carbohydrate. Its properties are similar to those of Reimann's "degraded" or "R" strains.

referred to the reaction between the ectoplasmic layer of specific carbohydrate and the type-specific anticarbohydrate antibodies; the species-specific agglutination of the non-S-producing strain may be referred to the reaction between the protein on the outside layer of the degraded bacterial cell and the species-specific antiprotein antibodies.

Antigenic Properties of the S-Producing and Non-S-Producing Strains of Pneumococci.

The differences in the antibody-invoking properties of the S-producing and non-S-producing strains were investigated by immunizing two series of rabbits with heated suspensions of the two sorts of pneumococci. The production of test-tube demonstrable antibodies was essentially the same as that reported by Reimann (5). The S-producing strain invoked the production of anticarbohydrate antibodies (type-specific agglutinins and type-specific S precipitins). The non-S-producing strain, on the other hand, was entirely devoid of the capacity to invoke the anticarbohydrate antibodies, and the serum obtained contained only the species-specific antiprotein antibodies and none of the type-specific antibodies.

The differences in the protective value of the serum yielded by immunization with the two strains are of more importance in the present paper than are the above serological reactions. The serum of animals immunized with the S-producing strain had a high passive protective value, regularly protecting mice against 0.01 cc. of virulent Type II pneumococci. In contrast, the serum of all animals immunized with the non-S-producing strain was entirely devoid of any protective power, although it possessed a high titre of species-specific antiprotein antibodies. Apparently, when the Type II pneumococcus cell loses its S-producing function, it no longer possesses the antigenic complex which invokes the production of passively protecting antibodies.

Active Immunization of Mice with S-Producing and Non-S-Producing Pneumococci.

Since there are frequent differences between passive and active antibacterial immunity (8), the following experiments were made to determine whether or not active immunity could be established by the vaccination of mice with non-S-producing pneumococci.

One series of ten mice was immunized with heat-killed vaccine prepared from the type-specific or S-producing strain, and another series with vaccine from the non-S-producing strain of pneumococci. Three courses of subcutaneous injec-

tions were given with a rest period of 1 week between courses; each course consisted of five doses of vaccine, each dose being given at 2 day intervals, equivalent to 0.15 cc. of broth culture. Tests for active immunity were made 11 days after the last vaccination, by the intraperitoneal injection of different amounts of Type II pneumococcus culture (10^{-6} to 10^{-3} cc.). Normal mice (not vaccinated) were injected with 10^{-6} cc. as virulence controls of the culture.

The results of the active immunity tests revealed the same differences in the antigenic properties of the two strains as those previously observed in the passive protection experiments. Although the protection was not uniform, some of the mice which had been actively immunized with the S-producing pneumococci survived the injection of doses of virulent Type II organisms equivalent to as much as one-thousandth cc. of culture.[2] In contrast to the series immunized with the type-specific organisms, none of the animals which had been vaccinated with the non-S-producing strain had acquired any detectable immunity and all were infected by doses of one-millionth cc. of the virulent culture. Apparently, therefore, the loss of the S-producing function of Type II pneumococci was accompanied by loss of the capacity to establish active immunity in mice, as well as by loss of the capacity to invoke passively protecting antibodies.

Absorption of Type II Antipneumococcus Serum with S-Producing and Non-S-Producing Pneumococcus Cells.

The results of preceding experiments have shown distinct differences in the antibody-invoking properties of the S-producing and non-S-producing pneumococci: the serum of animals immunized with the S-producing strain contained type-specific antibodies (agglutinins and S precipitins) and passively protected mice against Type II pneumococcus infection; in contrast, the serum of animals immunized with the non-S-producing strain contained no demonstrable type-specific anti-

[2] The irregularity in the protection of the mice in this series of vaccinated mice had no relation to the dose of pneumococci injected in the active immunity tests. Some individuals injected with 10^{-6} cc. and 10^{-5} cc. were infected, while others injected with 10^{-4} cc. and 10^{-3} cc. were protected. As reported in the following paper, the results of an investigation with a larger series of mice show that the above irregularities were due to differences in the immunity response of the individual mice.

bodies and did not protect mice. In view of these marked differences in the antibody-invoking properties of the two strains, experiments were made to determine if like differences existed in the capacity of the two strains to "absorb" or remove antibodies from type-specific immune serum.

A type-specific serum obtained by immunization of a rabbit with Type II pneumococcus vaccine was used in the absorption experiment. This serum contained the usual type-specific agglutinins, S precipitins, and passively protecting antibodies.

10 cc. of serum were placed in each of three tubes. 10 cc. of a salt solution suspension of heat-killed bacteria of the S-producing strain were added to one tube of serum; the same amount of a suspension of the non-S-producing pneumococci

TABLE I.

Absorption of Type II Antipneumococcus Serum with S-Producing and Non-S-Producing Pneumococci.

Type-specific antipneumococcus serum	Type II agglutinins	Precipitins for Type II soluble substance	Passively protecting antibodies
Unabsorbed	+	+	+
Absorbed with S-producing Type II pneumococci	0	0	0
Absorbed with non-S-producing pneumococci.	+	+	+

was added to the second tube of serum; an equal volume of salt solution was added to the third tube. The three mixtures were shaken for 5 minutes, then incubated for 2 hours at 37°C., and finally placed in the ice box for 12 hours. The tubes were then centrifuged to remove the bacteria. 10 cc. of the supernatant fluid from the above mixtures were then placed in each of three tubes and absorbed a second time by the same procedure.

After the removal of the bacteria used in the second absorption, the three sera ((1) unabsorbed control, (2) absorbed with S-producing pneumococci, (3) absorbed with non-S-producing pneumococci) were then tested for the presence of type-specific agglutinins, type-specific S precipitins, and type-specific protective antibodies. The agglutination and protection tests were made by the usual procedure. In the S precipitin tests, the bacteria-free filtrate of a young unautolyzed culture of Type II pneumococci was substituted for a solution of the purified carbohydrate. The results of the experiment are summarized in Table I.

The results of the absorption experiments (Table I) reveal the same general immunological differences as those obtained in the preceding

immunization experiments. Absorption with the heat-killed cells of the S-producing strain removes all the S precipitins, type-specific agglutinins, and protective antibodies, just as immunization with the bacterial cells of this strain invokes the production of these same type-specific antibodies. On the other hand, absorption with cells of the strain in which the S-producing function has been lost fails to remove any of these antibodies, just as immunization with cells of this strain failed to stimulate the production of type-specific antibodies. Hence, the pneumococcus cells which have lost their S-producing function have lost not only their property of invoking type-specific antibodies by antigenic stimulation in the animal body, but have also lost their capacity to "absorb" the same antibodies by type-specific antigen-antibody combinations in the test-tube.

Absorption of Type II Antipneumococcus Serum with Culture Filtrates of S-Producing and Non-S-Producing Pneumococci.

In the following experiment, Type II antipneumococcus serum was absorbed with filtrates of young broth cultures of the S-producing and non-S-producing pneumococci. It seemed that the results to be obtained by absorption of the serum with the filtrates should be comparable to those obtained by use of solutions of the chemically purified carbohydrate since the S substance is the only immunologically reactive substance which can be demonstrated in filtrates of young and unautolyzed broth cultures of Pneumococcus. (The S substance is liberated into the culture fluid in the early period of growth and if the fluids are filtered before cell disintegration has commenced, the filtrates are entirely free of any of the bacterial protein or other serologically reactive substances.) To obtain fluids containing the maximum amount of the soluble substance, the cultures were planted in glucose broth. The reaction of the cultures was observed closely and when a pH of approximately 6.5 was reached, the bacteria were removed by centrifugation, the reaction adjusted to pH 7.3, and the fluids filtered through a Berkefeld candle.

5 cc. of the antipneumococcus (Type II) serum were then placed in each of three tubes. 15 cc. of the filtrate from the S-producing culture were added to the first tube; 15 cc. of the filtrate from the non-S-producing culture were added to the second tube; an equal volume of broth was added to the third tube as a control. All the mixtures were shaken for 15 minutes, incubated for 3 hours at 37°C., and then placed in the ice box for 18 hours. The mixtures were then centrifuged and the clear supernatant fluids removed. A second absorption similar to the above was carried out with 10 cc. of the supernatant fluid of each of the above serum mixtures. Only slight precipitation occurred when the S-containing filtrate was

added to the serum which had received the preceding absorption treatment with this filtrate. After 3 hours at 37°C. and 18 hours at 5°C., the second series of mixtures was centrifuged and the supernatant fluids again removed. The supernatant fluids were then subjected to a third absorption by the described procedure. The mixtures were again centrifuged and the supernatant fluids removed. The supernatant fluids of the third absorption series were employed in the following tests. The sera at this stage had been diluted to about one-twenty-fifth of their volume, so that each cc. of the test sera contained approximately 0.04 cc. of serum and 0.96 cc. of broth, or culture filtrate.

Tests for type-specific agglutinins were made by the usual method with dilutions equivalent to 1/25 and 1/100 of the original serum. Two series of passive protection tests were made with different amounts of serum. One series of mice

TABLE II.

Absorption of Type II Antipneumococcus Serum with Culture Filtrates of S-Producing and Non-S-Producing Pneumococci.

Type II antipneumococcus serum	Passive protection					Type-specific agglutinins	
	Mice injected with Type II pneumococci					Serum dilutions	
	Amount of culture						
	10^{-2} cc.	10^{-3} cc.	10^{-4} cc.	10^{-5} cc.	10^{-6} cc.	1/25	1/100
Unabsorbed control...........	S	S	S	S	S	+	+
Absorbed with culture filtrate of S-producing pneumococci...	D	D	D	D	D	0	0
Absorbed with culture filtrate of non-S-producing pneumococci..................	S	S	S	S	S	+	+

S = survived 7 days. D = died 24 to 48 hours after injection.

received absorbed serum equivalent to 0.04 cc. of the original serum mixed with increments of broth culture ranging from 10^{-2} cc. to 10^{-6} cc. A second series of mice received the equivalent of 0.01 cc. of serum mixed with amounts of broth culture ranging from 10^{-3} cc. to 10^{-6} cc. The second series of protection tests was included to detect any incomplete absorption of protective antibodies which might not be recognized in tests with an excess of serum. Since the tests with the two amounts of serum gave identical results, only the results of the series with 0.04 cc. of serum are included in the summarized protocol presented in Table II.

Since the S substance was used for the absorption in the form of a solution in the filtrate of the S-producing strain, a slight excess of the carbohydrate remained in the serum mixture after the final absorption. Thus, it is obvious that a certain amount of the free carbohydrate was injected into the animals in the protection

tests with absorbed serum. In the absence of any evidence of an "aggressin"-like effect of small amounts of the S substance, we do not believe that this complicated the results of the experiment.

This experiment (Table II) shows that absorption with the filtrate of the S-producing strain removed all the type-specific agglutinins and also all the protective antibodies,[3] while the filtrate of the non-S-producing strain failed entirely to remove any of the type-specific antibodies and did not diminish the protective value of the serum. These results of the absorption with the culture filtrates were exactly analogous to the results (Table I) of the absorption with the heated whole cells of the two strains of pneumococci and, because of the simpler nature of the culture filtrates, furnish more direct evidence of the relation of Type II anti-S antibodies to protection.

The above results are also of interest from the standpoint of the identity of the type-specific agglutinin and the anti-S precipitin. Avery and Heidelberger's (3) contention that S precipitation and type-specific agglutination involve the same antigen-antibody combination, is supported by the fact that S precipitins are removed by absorption with suspensions of washed pneumococcus cells (Table I) and conversely by the fact that the type-specific agglutinins are removed by absorption with solutions of the S substance (Table II).

The studies of the soluble substance of Pneumococcus by Avery and Heidelberger (2, 3) and of the similar products of other bacteria by Zinsser and Mueller (9) have presented new conceptions of the importance of carbohydrates in immunology. From this point of view, the results in Table II are of general interest as an example of the removal of the protective antibodies of an antibacterial serum by absorption with solutions in which the only reactive substance is carbohydrate in nature.

DISCUSSION.

The elaboration of the soluble specific substance (carbohydrate) is a specialized function of the pneumococcus cell which is most highly

[3] It is important to note that this complete removal of protective antibodies by absorption with the S-containing fluid occurred in Type II immune rabbit serum. In similar experiments with Type I serum from an immune horse, protective antibodies were not completely absorbed when treated with culture fluid containing the Type I reactive carbohydrate.

developed in virulent strains. The loss of the function of S production is accompanied by changes in the immunological properties of pneumococci. As previously pointed out by Reimann (5), strains of pneumococci which no longer elaborate the specific carbohydrate, lose their type-specific serological properties and exhibit antigenic properties similar to those of solutions of pneumococcus protein (6, 7). In addition to the immunological changes manifested by differences in test-tube serological reactions, the loss of the S-producing function was also accompanied by marked changes in the antigenic properties involved in both passive and active protection of mice. The injection of S-producing pneumococci stimulated the production of passively protecting antibodies in rabbits and also established active immunity in mice. On the other hand, the similar injection of non-S-producing pneumococci neither invoked the production of passively protecting antibodies nor established active immunity.

The intimate relation of the type-specific antibodies to the antipneumococcus protection of mice was likewise evident in the results of absorption experiments. Absorption of Type II antipneumococcus serum with the heat-killed cells of S-producing pneumococci of homologous type removed the commonly recognized antibodies concerned in type-specific agglutination and in type-specific precipitation of solutions of the S substance. Serum from which these type-specific antibodies had been removed were completely devoid of passively protecting antibodies. On the other hand, when the Type II serum was subjected to the same absorption treatment with non-S-producing pneumococci, there was no detectable loss in the type-specific antibodies responsible for agglutination and S precipitation. This absorbed serum from which the type-specific antibodies had not been removed, possessed the same protective value as unabsorbed serum. More direct evidence of the relationship between the anticarbohydrate and the protective antibodies was furnished by the fact that absorption of Type II antipneumococcus serum with culture filtrates containing the S substance in solution removed the protective antibodies as completely as did the whole bacteria themselves. It is reasonable to believe that the removal of the protective antibodies was due to the reaction between the carbohydrate S substance and its type-specific antibody.

Although antipneumococcus immunity is type-specific and consequently the antibodies that confer it must also be type-specific, it is hazardous to conclude that Type II antipneumococcus protection is simply a function of the anticarbohydrate antibody. A number of reports have been made of the protective value of antipneumococcus serum which possesses none of the usual test-tube demonstrable antibodies. However, all our results indicate that the type-specific anticarbohydrate antibody is at least the dominant one involved in the protection of mice against virulent Type II pneumococci.

It is common experience (10) to find that the production of effective antipneumococcus serum requires the use of virulent pneumococci in the immunization. If the anti-S antibody is the one most prominently involved in Type II passive protection, the failure to obtain an effectively protecting serum by immunization with avirulent pneumococci would be explained not by the loss of the virulence of the bacteria employed as antigens, but by the fact that the avirulent bacteria no longer possess the antigenic complex (*SP* of Avery and Heidelberger (3)) which is required for the immunological production of type-specific antibodies.

SUMMARY.

The loss of the specialized function of S production by Type II pneumococcus was accompanied by a loss of the antigenic properties involved in both active and passive protection of mice. Absorption of Type II serum with S-producing pneumococci removed all the protective antibodies, as well as the type-specific agglutinins and S precipitins. The same absorption treatment of the serum by non-S-producing pneumococci failed entirely to remove the type-specific antibodies and did not affect the protective value of the serum. Absorption with bacteria-free culture fluids containing the reactive carbohydrate removed the protective antibodies as completely as absorption with the whole bacterial cells of type-specific strains. The results taken as a whole indicate that the antibodies involved in the usual protection of mice against Type II pneumococci are closely related, if not identical, to the specific anticarbohydrate precipitin.

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