IMMUNOLOGICAL REACTIONS OF THE ISOLATED CARBOHYDRATE AND PROTEIN OF PNEUMOCOCCUS.

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In a previous paper on the immunological relationships of the cell constituents of Pneumococcus (1) two chemically distinct substances, namely the carbohydrate and protein of the cell, were shown to be intimately concerned in the serological specificity of this organism. The facts recorded at that time related only to the reactions exhibited by these substances in antipneumococcus serum prepared by immunization with the whole bacterial cell. In the absence of final evidence as to the antigenic properties of these two cell constituents the conclusions drawn were limited to their serological behavior. The observations made then have since been confirmed, and in the present report are extended to include the distinctive character of the isolated substances when each is tested for its function as antigen.

Antigenic Properties of the Isolated Carbohydrate of Pneumococcus.—The results of chemical studies previously reported and of those now in progress leave little doubt that the so called soluble specific substance of Pneumococcus of the three fixed types is in each instance a polysaccharide (2). The essential fact of significance here is that from pneumococci of Types I, II, and III, three different polysaccharides have been isolated, each chemically as distinct as it is serologically type-specific. The present work brings further proof that this important constituent upon which the type specificity of the cell depends is itself, when dissociated from other cellular substances, devoid of antigenic properties.

Attempts to immunize rabbits by injections of pneumococcus carbohydrate have invariably yielded negative results. Up to the pres-

ent time intravenous and cutaneous inoculations of these protein-free polysaccharides in considerable amounts and in repeated doses have failed to stimulate any demonstrable antibodies in the sera of animals so treated. This fact is of especial interest since in dilutions as great as 1:5,000,000 this non-protein, non-antigenic substance in purified form has been found specifically reactive in antipneumococcus serum of the homologous type.

Antigenic Properties of Isolated "Nucleoprotein" of Pneumococcus.—The protein material precipitated by dilute acetic acid from filtered solutions of pneumococci shows the chemical reactions of nucleoprotein and mucoid. It is realized that the product so obtained can scarcely be considered an antigenic unit, consisting as it necessarily does of a mixture of different proteins. Although the chemical procedures used in the isolation and purification of the protein may have caused some alteration in its chemical nature, this change is apparently not different from that involved in the mere process of cell dissolution, since the results with the isolated protein are identical with those obtained with simple extracts and solutions of cellular substances made by freezing and thawing cell suspensions (3).

The protein substance was prepared by the method given in a previous report (1). In some instances instead of dissolving the washed bacterial cells with the minimum amount of bile necessary to effect complete solution, concentrated suspensions of organisms in 0.002 N NaOH were subjected to repeated freezing and thawing to disrupt the cell bodies. This thick solution of dissolved organisms was then diluted with salt solution to one-tenth the volume of original culture fluid and passed through a Berkefeld V filter. To this filtered solution N/1 acetic acid was added slowly and the mixture carefully shaken, flocculation of the protein occurring promptly and completely at a reaction faintly acid to litmus. The precipitated protein was separated by centrifugation, washed several times in distilled water, and redissolved in salt solution by adding 0.1N NaOH until the solution was faintly alkaline to litmus. Freshly prepared protein solutions obtained in this manner were used for the tests since the previous method of drying the material in vacuum after rapid washing in acetone and ether rendered the substance less soluble. Solutions of pneumococcus protein prepared by this method exhibit the usual qualitative color reactions for substances of this nature: positive biuret, Hopkins-Cole, Millon, xanthoproteic, and Molisch reactions. The hydrolyzed protein gives the purine reaction with Fehling's solution. The protein solutions were standardized on the basis of their nitrogen content.

Rabbits were immunized by intravenous injection of pneumococcus nucleoprotein in faintly alkaline solution. The injections were given on 6 consecutive days followed by a free interval of 6 days. Three series of treatments were given. The daily dose for the initial course of injections averaged about 5 mg., the total amount of protein given during immunization varying from 0.1 to 0.2 gm.

The sera of animals injected with pneumococcus protein were tested for (*a*) agglutinins, (*b*) precipitins, and (*c*) protective antibodies.

(*a*) *Agglutinins.*—Antiprotein sera obtained by immunization of rabbits with pneumococcus nucleoprotein do not contain type-specific agglutinins for pneumococci. Antisera against the protein isolated from pneumococci of Types I and II and Group IV have in no instance agglutinated either the homologous or heterologous strains. The

TABLE I.

Absence of Precipitins for Pneumococcus Carbohydrate in Antiprotein Serum of Homologous Type.

Immune sera.	Carbohydrate of Pneumococcus Type II (Lot 17).				
	1:2000	1:20,000	1:40,000	1:80,000	1:160,000
Type II antiprotein serum.....	−	−	−	−	−
Type II antipneumococcus serum.....	++	+++	++++	++++	++++

−, no precipitation; ++, flocculation; ++++, compact disc-like precipitate.

absence of specific agglutinins in these sera is most strikingly demonstrated when saline suspensions of heat-killed organisms are used in the reaction. Under these circumstances the autolytic ferments are inactivated so that dissolution of the cell with the resulting liberation of its protein into solution is prevented. When unheated organisms are used in the test a slight, finely granular precipitate may be frequently observed after the reaction mixtures have stood overnight in the ice box. Since this reaction is always delayed and is non-specific, occurring without quantitative differences in the presence of both homologous and heterologous types of pneumococci, it seems reasonable to assume that its occurrence under these conditions is referable to the interaction of antiprotein serum with the protein freed by cell autolysis.

TABLE II.

Precipitins for Pneumococcus Protein in Antiprotein Sera of Homologous and Heterologous Types.

Immune sera.	Rabbit No.	Protein of Pneumococcus Type I.						Protein of Pneumococcus Type II.					
		1:2000	1:4000	1:8000	1:16,000	1:32,000	1:64,000	1:2000	1:4000	1:8000	1:16,000	1:32,000	1:64,000
Type I protein.	1	+	+	+++	++	++	−	+	++	+++	+++	++	±
	2	++	+++	++++	+++	++	+	+	++	++++	+++	+++	+
" II "	3	+	+++	+++	+++	++	±	\	\	+++	+++	++	±
	4	+++	++++	++++	+++	++	±	\	\	++++	+++	++	+
Normal.	Control.	−	−	−	−	−	−	−	−	−	−	−	−

±, faint cloud; +, slight finely granular flocculation; ++, moderate precipitation; +++, marked precipitation; ++++, complete precipitation easily broken up; \, not done.

The results of many experiments demonstrate that immunization with the acid-precipitable pneumococcus protein, free from cell fragments, does not induce specific agglutinins for the intact cell.

(*b*) *Precipitins.*—Just as antiprotein sera contain no agglutinins for pneumococci of the specific types, so also do they lack precipitins for the specific carbohydrate derived from these organisms (Table I). The stimulation of antibodies having these specific reactions seems to be a function of the whole bacterial cell, since neither the carbohydrate nor the protein separately possesses this particular antigenic property.

As the result of immunization with the nucleoprotein from any strain of Pneumococcus, antibodies are formed which react with the same

TABLE III.

Cross-Precipitin Reactions of Antiprotein Serum of Pneumococcus Group IV with Solutions of Pneumococci of Different Types.

	Protein-containing solutions of pneumococci.				
Antiprotein serum of Pneumococcus Group IV.	Dilutions of antigen.	Type I.	Type II.	Type III.	Group IV.
	1:20	++	++	+++	+++
	1:40	+	++	++	++
	1:80	−	+	+	+
	1:160	−	−	−	±

protein fraction of other types of pneumococci. In Table II evidence is presented that antisera against the proteins of Pneumococcus Types I and II cross-react in precipitin tests with the isolated protein of both types. Further evidence of this reciprocal action is found in Table III, which indicates that the serum of a rabbit immunized to the protein derived from Pneumococcus Group IV precipitates protein extracts of dissolved pneumococci not only of the homologous group but also of the three fixed types.

(*c*) *Protection.*—Whether or not antiprotein serum prepared by immunization with the isolated nucleoprotein alone confers passive protection in animals against infection must remain undecided until further evidence is accumulated. In one instance the serum of a rabbit immunized with the protein of Type I pneumococcus protected

mice against 0.001 cc. of homologous culture of which 0.000,001 cc. proved fatal for the untreated controls. With the other lots of sera, slight or no protection against infection with homologous or heterologous pneumococci was afforded.

More recent study indicates that antiprotein serum obtained by immunizing with filtered solutions of dissolved pneumococci, although yielding antibodies reactive with the protein, confers no passive immunity on mice infected with virulent pneumococci (3).

Although the evidence is not final as to the protective value of antiprotein sera, sufficient experimental data are available to warrant the conclusion that such sera at best are much less effective than the antipneumococcus sera obtained by immunization with the whole bacteria.

SUMMARY.

The data presented in this paper clearly indicate that the isolated carbohydrate and nucleoprotein constituents of Pneumococcus differ both serologically and antigenically one from the other. Moreover, each of these fractions of the cell separately exhibits immunological properties distinct from those manifested by the whole organism of which they form a part.

The carbohydrate is a protein-free polysaccharide and as such is devoid of the property of stimulating antibodies. Although in the free state, dissociated from other cellular substances, it is non-antigenic, in this form it still retains the property of reacting specifically in antipneumococcus serum of the homologous type. Further, this non-protein constituent is not reactive with antiprotein serum. In other words, neither pneumococcus carbohydrate nor protein as separate antigen gives rise to antibodies with specific affinities for the carbohydrate or so called soluble specific substance of Pneumococcus.

The nucleoprotein of Pneumococcus, on the other hand, is antigenic. Immunization with this cell constituent gives rise to immune serum which precipitates solutions of pneumococcus protein without regard to the type from which it is derived.

The interrelations of the carbohydrate and protein of Pneumococcus as they exist in the intact cell to form the complete antigen, and the interpretation of the differences in the antigenic properties of the whole bacterium as contrasted with those of its component parts are reserved for discussion in a subsequent paper.

CONCLUSIONS.

1. The isolated carbohydrate of Pneumococcus is non-antigenic in the sense of stimulating antibody formation.

2. The isolated protein of Pneumococcus is antigenic: it induces the formation of antibodies which react with the nucleoprotein fraction of organisms of homologous and heterologous types.

3. The antiprotein sera do not agglutinate type-specific strains of Pneumococcus, or react with the carbohydrate derived from them.

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