CHEMO-IMMUNOLOGICAL STUDIES ON CONJUGATED CARBOHYDRATE-PROTEINS

V. The Immunological Specificity of an Antigen Prepared by Combining the Capsular Polysaccharide of Type III Pneumococcus with Foreign Protein

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(Received for publication, June 2, 1931)

The fundamental studies of Landsteiner and his coworkers (1) on complex antigens have established the important principle that the introduction into the protein molecule of a simple non-protein radical confers a new immunological specificity on the antigenic compound. Furthermore, this newly acquired specificity has been shown to depend upon the nature of the new chemical grouping thus introduced.

Previous studies from this laboratory (2) on the chemo-immunological properties of "synthetic antigens," prepared by combining a simple carbohydrate radical with protein, have shown that the specificity of the newly formed compounds is determined in each instance by the chemical individuality of the reactive carbohydrate, irrespective of the protein to which it is attached. Simple derivatives of glucose and galactose, which by themselves are non-antigenic will, when coupled to a common protein, stimulate the formation of antibodies that are specific for the particular sugar used. Antisera, produced by immunization with the conjugated sugar-proteins, invariably reflect the controlling influence of the carbohydrate on the specificity of the whole antigen. So sensitive is this chemo-specific effect, that mere differences in the spatial arrangement of the groups on a single carbon atom in two glucosides otherwise identical, were found sufficient to change completely the antigenic specificity of the respective compounds.

In order to test the possibility of synthesizing a specific antigen by

combining a bacterial carbohydrate with a foreign protein, the specific capsular polysaccharide of Type III Pneumococcus was purposely chosen, since in its purified form it contains no nitrogen and may be regarded as a definite chemical entity. Further, if results were obtained by the use of this particular bacterial polysaccharide, they would be the more significant, since the isolated pure substance alone has never been found to elicit antibodies in rabbits, and even the intact bacterial cells from which it is derived commonly fail to incite the formation of type-specific antibodies in these animals.

From a chemical point of view, the difficulty lay in synthesizing the appropriate derivative of the capsular polysaccharide. It must be one capable of coupling with protein on diazotization and one in which the chemo-specific groups of the bacterial sugar are not masked or destroyed in its preparation. The details of the chemical methods used in the synthesis of the aminobenzyl ether of the capsular polysaccharide of Type III Pneumococcus have been described in the preceding paper (3). The diazonium derivative of the polysaccharide was coupled with globulin prepared from horse serum, and the newly formed compound was tested for antigenicity by repeated intravenous injection in rabbits. The sera of the immunized animals were tested for the presence of type-specific agglutinins and protective antibodies against encapsulated, virulent strains of Type III Pneumococcus and for precipitins against the type-specific capsular polysaccharide and its aminobenzyl derivative. The treated rabbits were subsequently infected with a rabbit-virulent strain of Type III Pneumococcus to determine whether, as a result of immunization, they had acquired active immunity against Type III infection.

In evaluating the immunological findings presented in this paper, the fact should be borne in mind that the antigen used in the experiments has in common with the Type III Pneumococcus only the specific capsular polysaccharide, and that the protein with which it is conjugated is of widely remote biological origin.

EXPERIMENTAL

Methods

Immunization.—Rabbits were immunized by the intravenous injection of from 1 to 2 cc. of solutions of the conjugated carbohydrate-protein antigen daily for

six doses, and the course of injections was repeated a second time after a rest period of 7 days. 8 days after the last injection the rabbits were bled and the serum tested for type-specific pneumococcus antibodies.

Antigen.—The antigen, prepared as described in the preceding paper, was preserved by the addition of 0.25 per cent tricresol. Each preparation was standardized on the basis of nitrogen content, so that 1 cc. of the solution contained 5 mg. of protein. This method, however, does not indicate the amount of bound carbohydrate and hence is not an accurate measure of the effective antigenic complex. The protein, to which the aminobenzyl ether derivative of Type III polysaccharide was bound by the diazo reaction, was globulin prepared from horse serum by precipitation with ammonium sulfate.

Technic of Immunity Reactions.—In the precipitin reactions, the immune serum was diluted in the proportion of two parts of serum to three of salt solution and 0.5 cc. of this dilution containing 0.2 cc. of original serum was added to 0.5 cc. of varying dilutions of reacting substance. The final concentration of precipitinogen in the reaction mixture is shown in the protocols. The protection tests were done by the usual technic; mice were injected intraperitoneally with 0.2 cc. immune serum together with varying dilutions of an actively growing culture of Pneumococcus. The dilutions were so made that the total volume injected was 1 cc. in all instances.

I. Type-Specific Antipneumococcus Antibodies

1. Precipitins.—The sera of rabbits immunized with the Type III "synthetic antigen" were tested for the presence of precipitins for the original capsular polysaccharide and for the aminobenzyl ether derivative used in preparing the antigen. The introduction of the para-aminobenzyl radical into the polysaccharide molecule did not destroy the immunological specificity of the derivative, since the latter reacted with Type III antipneumococcus horse serum in high dilutions, showing a specific reactivity comparable to that of the original polysaccharide from which it was derived. It was of interest, therefore, to determine whether this specific carbohydrate derivative, when bound with protein, would stimulate the formation of precipitins reactive not only with the amino derivative but also with the polysaccharide itself, when both substances were used in the free, soluble form.

The precipitin reactions of immune rabbit serum with the original and modified form of the Type III capsular polysaccharide are compared in Table I. The results show that the serum of a rabbit immunized with the "synthetic antigen" reacts with the native polysaccharide and with its aminobenzyl ether in equally high dilutions.

Similarly, as previously pointed out, the serum of a horse immunized with the intact bacterial cells containing the natural Type III antigen precipitates the polysaccharide derivative as well as it does the polysaccharide itself. The evidence is, therefore, that the specifically reactive groups of the polysaccharide have not been chemically masked in forming the new derivative, and that when this specifically reactive carbohydrate is chemically bound with protein to form a new antigen, it orients the immune response as specifically as does the polysaccharide itself in the form in which it exists as a natural antigen in the encapsulated cells.

TABLE I

Precipitins for the Capsular Polysaccharide of Type III Pneumococcus in Serum of Rabbits Immunized with Type III Synthetic Antigen

Dilution of carbohydrate	Purified preparation of Type III polysaccharide	Aminobenzyl ether of Type III polysaccharide
1:100,000	++±	+++
1:200,000	+++	+++
1:400,000	++±	+++
1:800,000	+±	+
1:1,600,000	+	±

+++ = complete precipitation with compact disc.
++ = diffuse turbidity.
+ = slight turbidity.

2. *Agglutinins.*—The sera of rabbits, prepared by serial injections of the chemically combined antigen, specifically agglutinate encapsulated strains of Type III pneumococci.

The results of the agglutination reactions, as illustrated in Table II, show that while the titre of agglutinins is not high, the serum is type-specific in that it agglutinates only Type III pneumococci, and fails to react with organisms of Type I and Type II. The fact that the immune serum does not agglutinate the non-encapsulated R forms of Pneumococcus was to be expected, since the antigen contains none of the somatic constituents commonly present in all pneumococci. The lack of these cellular substances in the artificial antigen is reflected in the serum by the absence of the species-specific antibodies, which

are ordinarily present when the whole cell is used as the immunizing agent.

TABLE II

Specificity of Agglutination Reaction with Serum of Rabbit Immunized with Type III Synthetic Antigen

Pneumococcus	Immune serum			
	1:5	1:10	1:20	1:40
Type I	−	−	−	−
Type II	−	−	−	−
Type III	++++	+++	+	±
"R" strain*	−	−	−	−

++++ = complete agglutination with disc formation.
− = no reaction.
* Derived from Pneumococcus Type II.

TABLE III

Protective Action in Mice of Sera of Rabbits Immunized with Type III Synthetic Antigen

Pneumococcus Type III	Immune rabbit sera (0.2 cc.)								Virulence controls No serum	
	(a)		(b)		(c)		(d)			
cc.										
0.01	−	−	−	−	D 22	D 22	D 7	D 24	−	
0.001	−	−	−	−	S	S	S	S	−	
0.0001	S	S	S	S	S	S	S	S	−	
0.00001	S	S	S	S	S	S	S	S	−	
0.000001	S	S	S	S	S	S	S	S	D 42	D 42
0.0000001	−	−	−	−	−	−	−	−	D 42	D 45
0.00000001	−	−	−	−	−	−	−	−	D 75	D 45

S = survival.
D = death of animal, the numeral indicates the number of hours elapsing before death.
− = not done.

3. Protective Antibodies.—To ascertain whether passive immunity against pneumococcus infection could be conferred on mice by the injection of the serum of a rabbit immunized with the artificial Type

III antigen, protection tests were carried out by the technic described. A constant amount of immune serum (0.2 cc.) was injected intraperitoneally into mice together with varying quantities of a virulent culture of Pneumococcus.

The results given in Table III show that the immune rabbit sera were effective in protecting mice against 10^{-3} cc. of a virulent culture

TABLE IV

Type Specificity of Protective Antibodies in the Sera of Rabbits Immunized with Type III Synthetic Antigen

Immune serum	Amount of culture	Pneumococcus		
		Type I	Type II	Type III
cc.	*cc.*			
0.2	10^{-3}	—	—	S S
0.2	10^{-4}	D 28 D 28	D 28 D 28	S S
0.2	10^{-5}	D 46 D 28	D 28 D 46	S S
0.2	10^{-6}	D 28 D 46	D 28 D 28	S S
Virulence controls	10^{-6}	D 46	D 28	D 46
	10^{-7}	D 72	D 46	D 46
	10^{-8}	S†	D 46††	D 46†††

S = survival.

D = death of animal; the numeral indicates the number of hours elapsing before death.

— = not done.

† The number of colonies developing in blood agar seeded with this inoculum = 0.

†† The number of colonies developing in blood agar seeded with this inoculum = 3.

††† The number of colonies developing in blood agar seeded with this inoculum = 2.

of Type III Pneumococcus, of which 10^{-8} cc. was invariably fatal when injected alone into untreated control animals. Although the maximum degree of protection afforded by the serum was not high, the special interest of these experiments lies in the fact that the protective antibodies were produced in response to a combined antigen containing a specifically reactive carbohydrate which by itself fails to evoke any immune response in rabbits.

The specificity of the protective antibodies in the sera of rabbits immunized with the Type III synthetic antigen is shown in Table IV.

The protective action of the immune serum is strictly type-specific. Mice receiving the immune serum survived infection with Type III Pneumococcus, but succumbed promptly to minimum doses of Type I or Type II culture. The marked specificity of the protection afforded by the serum is further evidence that the antibodies concerned in the reaction derive their specificity from the polysaccharide component of the whole antigen. The protein constituent of the antigen appears to contribute nothing to the antibacterial properties of the serum, except in so far as it renders the carbohydrate antigenic.

4. Active Immunity.—Of the series of rabbits injected at various times with effective preparations of antigen, none have failed to respond with the formation of type-specific antibodies. It was of interest, therefore, to ascertain whether rabbits in the sera of which Type III Pneumococcus antibodies were demonstrable had acquired active immunity against infection with this organism. In order to determine this point, several of the treated animals were subsequently infected with multiple lethal doses of a virulent culture of Type III Pneumococcus.

11 days after the last injection of antigen, four immunized rabbits whose serum contained type-specific antibodies were infected by the intradermal injection of 0.2 cc. of an 18 hour blood broth culture of a rabbit-virulent strain of Type III Pneumococcus. The virulence of this strain, maintained by repeated rabbit passage, was such that 0.00001 cc. of the culture alone injected into the skin proved fatal within 72 hours.

Four rabbits, which had received repeated injections of the "synthetic antigen," were reserved and later tested for active immunity. All four of these rabbits survived infection. In the two animals which had the highest titre of type-specific antibodies in their serum, the infection ran a practically afebrile course with only a slight inflammatory reaction at the point of inoculation; in the other two rabbits, the temperature remained relatively low and the skin lesion was not severe. At no time during the course of the infection were pneumococci present in cultures of the blood of the immunized rabbits, whereas in the normal controls the bacteremia invariably increased up to the time of death.

The results demonstrate that an antigen prepared by combining the Type III capsular polysaccharide with an animal protein is capable of inducing in rabbits an active immunity against infection with a virulent culture of Type III Pneumococcus. The only constituent of Pneumococcus in the artificial antigen is the capsular polysaccharide. Since this carbohydrate alone is non-antigenic in rabbits, the antibacterial immunity induced by the combined antigen can be ascribed only to the antigenicity acquired by the polysaccharide through combination with the foreign protein. The results bring evidence that an effective, active immunity can be developed in which the only antibacterial antibodies formed are those directed against the capsular component of the pneumococcus.

II. Antiprotein Antibodies

In the conjugated antigen, the protein used in combination with the Type III capsular polysaccharide was globulin derived from normal horse serum. The total protein of the antigen was estimated by determining the nitrogen content of the various preparations. This method of standardization, while affording a measure of the total globulin present, does not indicate the amount of protein that is bound with the polysaccharide to form the effective type-specific antigen. The sera of immune rabbits invariably contained not only type-specific antibodies for Type III Pneumococcus, but also specific precipitins for the globulin of horse serum.

The presence in immune rabbit serum of precipitating antibodies for the foreign protein used in preparing the antigen is shown in Table V. The antiprotein precipitins evoked by the combined antigen may be attributed to one or both of two possibilities. In the first place, despite attempts to remove the excess of unbound globulin from solution, it is possible that sufficient protein remains free and uncombined with the polysaccharide to function independently as antigen. It is also possible, however, that even in the absence of any free globulin, the combined antigen itself may give rise to two qualitatively different antibodies, each specifically related to the corresponding component of the antigenic complex. This explanation of the concurrence of two distinct varieties of antibodies in the immune serum involves the assumption, that while the carbohydrate

has acquired antigenicity through combination with the globulin, the protein molecule itself has not lost its own specific antigenic property. According to this view, a single compound of carbohydrate and protein may possess the dual antigenic property of stimulating two separate and specific antibodies; one evoked by the newly acquired antigenicity of the polysaccharide in union with globulin, and the other stimulated by the protein molecule itself in which the chemo-specific groups essential for antigenicity have not been masked in the linkage with the carbohydrate.

TABLE V

Antiglobulin Precipitins in Sera of Rabbits Immunized with Type III Synthetic Antigen

Immune sera	Globulin (horse serum)*			
	1:20,000	1:40,000	1:80,000	1:160,000
(*a*)	+++	+++	++	±
(*b*)	+++±	+++	++	+
(*c*)	++++	+++±	+±	±
(*d*)	++++	+++	+±	+
(*e*)	+++±	++±	+	±

++++ = flocculent precipitation, clear supernatant.

\+ = slight, diffuse turbidity.

* The diazonium derivative of the capsular polysaccharide of Type III Pneumococcus was coupled with globulin from horse serum.

Of the two explanations, the first, namely that the free globulin in the antigenic mixture accounts for the antiprotein antibodies, is the simpler and perhaps the more likely one. However, the second concept, which ascribes a dual antigenicity to the sugar-protein, affords an immunologically interesting and theoretically possible explanation.

DISCUSSION

In the present state of our knowledge it would be hazardous to predict the precise conditions under which complex carbohydrates by themselves may function as antigens. The present study simply defines certain experimental conditions under which the capsular polysaccharide of Type III Pneumococcus, in chemical union with a

foreign protein, is rendered specifically antigenic in a particular species of animals, in which the carbohydrate alone has never been found to incite antibody formation. The "synthetic antigen" elicits in rabbits a type-specific antipneumococcus response, which neither one of its constituents alone is capable of inciting when injected singly into these animals.

In order to effect the chemical union, it was first necessary to synthesize a derivative of the bacterial carbohydrate that could be diazotized and coupled to protein by means of the diazo linkage. This was accomplished by forming the aminobenzyl ether of the polysaccharide, a derivative which fulfills the chemical requirements while still retaining the serological specificity of the original carbohydrate.

Antisera prepared by immunization with the combined antigen contain type-specific antibodies which precipitate the Type III capsular polysaccharide, agglutinate pneumococci of the homologous type, and protect mice against infection with virulent strains of Type III Pneumococcus. The fact that, in the present instance, the antipneumococcus response is produced by an antigen known to contain but a single component of the bacterial cell, indicates the unity of the antibodies participating in the type-specific reactions of precipitation, agglutination, and protection, and relates the specificity of these antibodies to that of the capsular polysaccharide in the reactive part of the antigenic molecule.

CONCLUSIONS

1. Type-specific antipneumococcus immunity has been induced in rabbits by immunization with antigen prepared by combining a specific derivative of the capsular polysaccharide of Type III Pneumococcus with globulin from horse serum.

2. Rabbits immunized with this antigen acquire active immunity against infection with virulent Type III pneumococci.

3. The sera of the immune rabbits contain type-specific antibodies which precipitate the Type III capsular polysaccharide, agglutinate Type III pneumococci, and specifically protect mice against Type III infection.

4. The experimental data are discussed with reference to: (1) the concurrence in the immune sera of type-specific antibodies for Pneu-

mococcus and precipitins for horse globulin; (2) the determining influence of the capsular polysaccharide on the specificity of the antigen as a whole; (3) the unity of the type-specific precipitins, agglutinins, and protective antibodies induced by a single component of the pneumococcus in chemical union with an unrelated, animal protein.

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