STUDIES ON ANTIBACTERIAL IMMUNITY INDUCED BY ARTIFICIAL ANTIGENS

I. Immunity to Experimental Pneumococcal Infection with an Antigen Containing Cellobiuronic Acid

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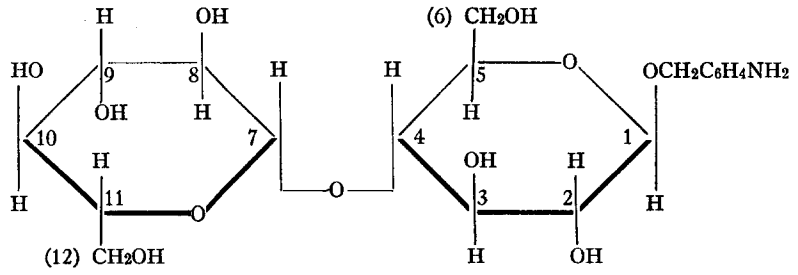
The results of experimental studies on artificial antigens containing the azobenzyl glycosides of glucuronic, galacturonic, and cellobiuronic acids indicate that the hexose uronic and aldobionic acids have an important function in determining the immunological characteristics of certain of the specific polysaccharides of encapsulated microorganisms (1). Azoproteins containing these uronic acids have the property of precipitating in high dilutions in antipneumococcal sera of various types, whereas antigens containing the azobenzyl glycosides of the corresponding aldoses show little or no serological activity. That the hexose uronic acid antigens actually combine with and precipitate the type specific polysaccharide antibodies has been demonstrated in a number of ways. It is apparent, therefore, that the artificial hexose uronic acid antigens possess certain of the serological characteristics of the immunologically active pneumococcus polysaccharides themselves. Despite this similarity, however, it has thus far been impossible to induce antibacterial immunity by injecting animals with the glucuronic or galacturonic acid antigens. Attempts to induce immunity to Type III pneumococcal infections in mice, rabbits, goats, and horses with an azoprotein containing glucuronic acid have all been unsuccessful. The reason probably resides in the fact that glucuronic acid alone does not approximate closely enough the chemical structure of the more complex building stone of the Type III pneumococcus polysaccharide, cellobiuronic acid (2).

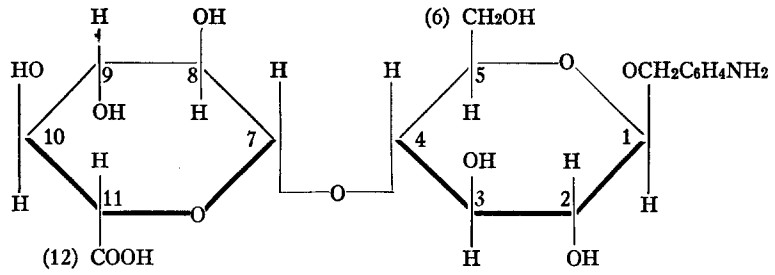
Recently it has been shown that an azoprotein containing cellobiuronic acid simulates much more closely the serological character-

istics of the capsular polysaccharide of Type III Pneumococcus than does one containing glucuronic acid (1 *c*). These observations have led to the belief that in order to induce Type III antipneumococcal immunity in experimental animals with an artificial antigen, it is not necessarily essential to have as the immuno-specific group the long chained type specific polysaccharide (3), but that the building stone from which it is constituted, namely the aldobionic acid, should suffice. In accepting this point of view it must be borne in mind that the polysaccharides, biologically active and otherwise, are simpler entities than are the antigenic proteins. The carbohydrates may in general be regarded as constituted from a fundamental pattern unit of one or more simple saccharides combined in glycosidic union to form a long chained macromolecule. Whether in artificially compounded antigens the integrity of the bacterial polysaccharide molecule is essential for the expression of type specificity and the capacity to induce antibacterial immunity, or whether the pattern unit, which in the case of the Type III Pneumococcus is cellobiuronic acid, will suffice, is the subject of the present investigation.

Two artificial azoprotein antigens have therefore been prepared, one containing the azobenzyl glycoside of the disaccharide cellobiose, the other the corresponding glycoside of cellobiuronic acid, the pattern unit of the Type III pneumococcus specific polysaccharide. The structural relationship of these two glycosides is represented by the following formulae:



p-aminobenzyl β-cellobioside



p-aminobenzyl β-cellobiuronide

These two substances differ only in the grouping occupying the 12th position which in the cellobiose is a primary alcohol grouping (CH_2OH) and in the cellobiuronide a carboxyl group (COOH). From the following account it will be seen that this slight difference in chemical constitution confers upon each antigen vastly different immunological properties.

EXPERIMENTAL

Methods

Immunization.—Two groups of rabbits weighing from 2 to 2.5 kilos were immunized respectively by the intravenous injection of 1 cc. of a sterile 0.5 per cent solution of the cellobiose and cellobiuronic acid antigens. The latter were prepared by combining the diazotized derivatives of the *p*-aminobenzyl glycosides of cellobiose and cellobiuronic acid to horse serum globulin as previously described (1 *c*). The animals received six daily doses of antigen and after a rest period of 7 days, the course of injections was repeated a second time. When necessary a third course of immunization was given. 7 days after the last injection the animals were bled from the ear and the sterile serum kept without preservative. In the tables the immunizing antigens will be referred to as cellobiose-globulin and cellobiuronic acid-globulin.

Technique of Immunity Reactions.—In the precipitin reactions, test antigens were prepared by combining the diazotized glycosides to the protein of chicken serum in order to avoid protein cross-reactions. In the agglutination reactions the antiserum to be tested was diluted with the appropriate quantity of sterile saline and an equal quantity of freshly prepared suspensions of heat-killed (70°) pneumococci of the specific type indicated in the protocols was added. All tubes were incubated at 37° for 2 hours and readings made after 24 hours in the ice chest.

The protection tests were done by the conventional technique; mice were injected intraperitoneally with 0.2 cc. of immune serum together with graded amounts of virulent cultures of pneumococci. The dilutions were so made that

in all instances the total volume injected was 1 cc. Only those antisera having maximal precipitin titre for the homologous test antigen were used in mice, since antisera of lower titres failed to show appreciable protective action against virulent pneumococci. Following the course of intravenous injections the rabbits were tested for active immunity by the intradermal method of Goodner (4), using a culture of a rabbit virulent strain of Type III Pneumococcus, 0.001 cc. of which killed normal animals within 48 hours. The extent and character of the lesions, as well as the temperature of the animals, were recorded daily. All animals were observed for a period of 18 days before terminating the experiments.

RESULTS

Precipitins.—The immunization of the rabbits with the azoprotein antigens containing cellobiose and cellobiuronic acid was followed by means of the precipitin test. Two antisera obtained from each of two groups of rabbits which had received the cellobiuronic acid antigen were chosen for further investigation. Two cellobiose antisera likewise obtained from two groups of each of three rabbits were used in the immunological studies. All of these antisera yielded a marked precipitate with high dilutions of homologous test antigens.

Neufeld "Quellung" Reactions.—Using the standard technique for the Neufeld reaction it was found that a young actively growing culture of Type III Pneumococcus when mixed with cellobiuronic acid antiserum showed a typical and unmistakable swelling of the capsule indistinguishable from the Neufeld reaction produced by Type III anti-pneumococcus rabbit serum. The specificity of this reaction is the more striking since antisera to the cellobiose antigen failed to produce swelling of the capsule of Type III pneumococci. No swelling of the capsules of Types II and VIII pneumococci could be observed when the respective organisms were tested with cellobiose or cellobiuronic acid antisera. This point will be discussed further in the section dealing with the protective action of these sera.

Agglutinins.—The sera of rabbits injected with the cellobiose and cellobiuronic acid antigens were tested for agglutinins with heated suspensions of Types II, III, and VIII pneumococci. The results of typical experiments are given in Table I. From the results given in Table I it can be seen that the sera of rabbits immunized with the cellobiose antigen failed to agglutinate, in the range of dilutions used, any of the types of pneumococci tested. On the other hand, the

cellobiuronic acid antisera in high dilutions agglutinated specifically Type III pneumococci but not the organisms of Types II and VIII. These experiments were carefully controlled in that the serum of the same animals obtained before immunization was similarly tested and in each instance found to be wholly devoid of specific antibodies for Type III pneumococci.

From the results of these experiments it can be concluded that the antisera of rabbits immunized with the artificial cellobiuronic acid antigen contain antibodies which cause swelling of the capsules and agglutination of Type III pneumococci, whereas the cellobiose antisera show neither of these properties. It is apparent, therefore, that the

TABLE I

Agglutination of Types II, III, and VIII Pneumococci in Cellobiose and Cellobiuronic Acid Antisera

Antiserum prepared by immunization with	Pneumococcus Types	Final dilution of serum						
		1:5	1:10	1:20	1:40	1:80	1:160	1:320
Cellobiose-globulin	II	0	0	0	0	–	–	–
	III	±	0	0	0	–	–	–
	VIII	0	0	0	0	–	–	–
Cellobiuronic acid-globulin	II	0	0	0	0	–	–	–
	III	++	++	+++	+++	++	+	±
	VIII	±	0	0	0	0	0	0

conversion of the primary alcohol group on the 12th carbon atom of cellobiose to the carboxyl group confers upon the cellobiuronic acid a new and important immuno-chemical function.

Protective Antibodies: A. Cellobiuronic Acid Antiserum.—It has been found that the capsular polysaccharides of Types III and VIII pneumococci both contain cellobiuronic acid as an important constituent of the molecule (5). In order to determine whether sera of rabbits immunized with the artificial cellobiuronic acid antigen will confer passive immunity on mice against infection with these types of pneumococci, protection tests were performed by the technique described. For purposes of comparison tests against infection with Type I pneumococci were included in this experiment as well. Since the

capsular polysaccharide of Type I Pneumococcus bears no structural similarity to that of Types III or VIII, one would not anticipate any protective action of the cellobiuronic acid antiserum against infection with organisms of Type I.

The results of the protection experiments given in Table II show that the serum of a rabbit immunized with the artificial cellobiuronic acid antigen is effective in protecting mice against infection with 10,000 and 1000 minimal lethal doses of Types III and VIII pneumo-

TABLE II

*Protective Action of Anticellobiuronic Acid Rabbit Serum against Pneumococcus Infection in Mice**

Amount of culture	Pneumococcus								
	Type I		Type III			Type VIII		
cc.								
10^{-3}	–	–	D 48	D 48	S	–	–	–
10^{-4}	–	–	S	S	S	D 44	D 72	S
10^{-5}	D 40	D 40	S	S	S	S	S	S
10^{-6}	D 40	D 48	S	S	S	S	S	S
Virulence controls† (no serum)								
10^{-6}	D 40		D 32			D 28		
10^{-7}	D 48		D 48			D 28		
10^{-8}	D 48		D 48			S		

* The serum of a rabbit immunized with the first preparation of cellobiuronic acid antigen and showing the highest precipitin titre for the homologous test antigen was chosen for this experiment.

† The number of colonies developing in blood agar, seeded with the 10^{-7} and 10^{-8} dilutions were in all instances counted (Tables II to IV).

cocci respectively. As was anticipated, the anticellobiuronic acid serum failed to protect against infection with virulent Type I pneumococci.

These experiments proved so striking that it was thought advisable to repeat them. Consequently an entirely new lot of the *p*-aminobenzyl glycoside of cellobiuronic acid was synthesized and the experiments repeated. The second preparation of antigen was administered as in the previous experiment to a group of three normal rabbits. After three courses of immunization one animal in this group failed to

show cellobiuronic acid antibodies, a second gave a moderate antibody response, whereas the serum of the third animal showed the presence of precipitins in high titre and was used in the following protection tests.

This serum was tested in mice for the presence of protective antibodies against Pneumococcus Types II, III, and VIII and the results of these experiments are given in Table III. The experiments were controlled by including a group of mice which received virulent organisms together with the serum of the same rabbit before immunization with the cellobiuronic acid antigen was begun.

The results presented in Table III confirm the observations recorded in Table II and in addition show that the cellobiuronic acid antiserum affords protection against infection with Type II pneumococci as well as with Types III and VIII organisms. This result clearly demonstrates that the artificial antigen containing the azobenzyl glycoside of cellobiuronic acid stimulates in rabbits the formation of antibodies capable of conferring passive immunity on mice against infection with a number of different types of virulent pneumococci. The significance of this finding will be discussed later.

B. Cellobiose Antiserum.—It will be recalled that the chemical structure of the two saccharides, cellobiose and cellobiuronic acid, is identical save for the grouping occupying the 12th position in each. Antigens containing the azobenzyl glycosides of these two saccharides give rise in rabbits to antibodies which show some serological crossing, yet are quite specific as shown by inhibition tests (1 *c*). In the present investigation it has been found that the antiserum elicited by the cellobiuronic acid antigen agglutinates Type III pneumococci and causes a definite swelling of the capsule. The antiserum to the cellobiose antigen, on the other hand, exhibits neither of these phenomena.

In order, therefore, to ascertain whether the cellobiose antiserum will confer passive protection against pneumococcal infections, the most potent cellobiose antiserum was tested in mice with virulent cultures of Types II, III, and VIII pneumococci by the method described. In no instance was any protective action observed. The results of these experiments, which are given in Table IV, again demonstrate the wide variance in immunological function of the anti-

bodies elicited by an antigen containing the disaccharide as opposed to the immune bodies evoked by the aldobionic acid antigen.

Active Immunity: A. Rabbits Injected with Cellobiose Antigen.— To ascertain whether the rabbits injected with the cellobiose antigen had acquired active immunity, six animals were infected, 12 days after the last injection of antigen, by the intradermal inoculation of

TABLE III

*Protective Action of Anticellobiuronic Acid Rabbit Serum against Pneumococcus Infection in Mice**

Amount of culture	Pneumococcus					
	Type II		Type III		Type VIII	
cc.						
10^{-2}	D 72	S	D 24	D 24	—	—
10^{-3}	S	S	D 72	S	D 24	D 24
10^{-4}	S	S	S	S	D 40	D 72
10^{-5}	S	S	S	S	S	S
10^{-6}	—	—	—	—	S	S
Controls†						
10^{-6}	D 40		D 40		D 40	
10^{-7}	D 24		D 48		D 40	
10^{-8}	D 48		D 48		D 40	
Virulence controls (no serum)						
10^{-6}	D 40		D 40		D 40	
10^{-7}	D 24		D 48		D 40	
10^{-8}	D 40		D 48		S	

* The serum of a rabbit immunized with the second preparation of cellobiuronic acid antigen and showing the highest precipitin titre for the homologous test antigen was chosen for this experiment.

† Mice received 0.2 cc. of serum of rabbit before immunization with cellobiuronic acid antigen was begun.

0.2 cc. of a blood broth culture of a rabbit virulent strain of Type III Pneumococcus. The virulence of the culture was such that 0.001 cc. injected intradermally killed normal control rabbits within 48 hours. The animals which had previously received the cellobiose antigen promptly developed massive edematous and necrotic lesions following infection and succumbed within 48 to 60 hours.

B. Rabbits Injected with Cellobiuronic Acid Antigens.—Four of the rabbits which had received the cellobiuronic acid antigen were likewise tested for active immunity. The intradermal inoculation of virulent Type III organisms was made 12 days after the last injection of antigen. All four animals developed marked lesions and ran a febrile course. In each instance save one, however, the lesions were smaller and less edematous than in the normal controls or in the animals which had received the cellobiose antigen. Three of the infected rabbits recovered from the dermal infection. One rabbit died within 72 hours.

TABLE IV

*Protective Action of Anticellobiose Serum against Pneumococcus Infection in Mice**

Amount of culture	Pneumococcus					
	Type II		Type III		Type VIII	
cc.						
10^{-5}	D 28	D 45	D 45	D 45	D 45	D 45
10^{-6}	D 47	D 72	D 47	D 47	D 45	D 45
Virulence controls (no serum)						
10^{-6}	D 45		D 45		D 40	
10^{-7}	D 45		D 45		D 45	
10^{-8}	D 45		D 45		D 45	

* The serum of a rabbit immunized with cellobiose antigen and showing the highest precipitin titre for the homologous test antigen was chosen for this experiment.

The results of these experiments indicate clearly that rabbits immunized with the cellobiuronic acid antigen acquire definite resistance to intradermal infections with a virulent strain of Type III Pneumococcus. Animals injected with the cellobiose antigen, on the other hand, show no resistance whatsoever.

DISCUSSION

From the results of our immuno-chemical studies on uronic acid antigens, the concept has gradually evolved that it might be possible to confer on experimental animals immunity to pneumococcus infec-

tion with an artificial antigen containing a simple saccharide as the immuno-specific group instead of the more complex bacterial polysaccharide itself. Earlier studies showed that artificial antigens containing the azobenzyl glycosides of glucuronic and galacturonic acids, though reactive in antipneumococcal sera, failed to stimulate in various species of experimental animals immunity to pneumococcal infections (1 *b*). The reason for this failure may be attributed to the fact that the hexose uronic acids do not approximate closely enough in structure the aldobionic acids which constitute the fundamental building stones of certain of the type specific polysaccharides of bacterial origin. The structural unit of the Type III pneumococcus polysaccharide is cellobiuronic acid (2 *a*). This aldobionic acid is unusually suited for testing the hypothesis set forth above, since much of the basic research for such a study already has been accomplished, and the acid itself is readily procured from the acid hydrolysis products of the bacterial polysaccharide. From the results of the present investigation it has been proven beyond question that the aldobionic acid, functioning as the immuno-specific group of an artificial antigen, evokes in rabbits antibodies which have many properties in common with those elicited by an antigen containing the more complex capsular polysaccharide.

In a communication presented some years ago from this laboratory (3), it was shown that an artificial antigen containing the azobenzyl ether of the Type III capsular polysaccharide evoked in rabbits antibodies which specifically agglutinated Type III pneumococci, precipitated the homologous specific polysaccharide, and protected mice against infection with Type III organisms. Not only does the cellobiuronic acid antiserum precipitate the Type III capsular polysaccharide, when combined with egg albumin (1 *c*), and agglutinate Type III organisms, but the sera of animals immunized with the cellobiuronic acid antigen likewise confer passive protection on mice against infection with virulent Type III pneumococci.

It should not be inferred, however, that the antibodies evoked by the polysaccharide antigen or by heat-killed Type III pneumococci are identical with those elicited by the cellobiuronic acid antigen. The results of the specific inhibition tests presented in the previous communication (1 *c*) clearly demonstrate that the polysaccharide and cellobiuronic acid antibodies are similar but not identical since they

fail to show a complete reciprocal relationship. Furthermore the results of the experimental studies presented in this communication have brought forth a new and important principle. Whereas the antigen containing the complex bacterial Type III pneumococcus carbohydrate gives rise to antibodies which are type specific, those elicited by the antigen containing the pattern unit, or aldobionic acid show a broader specificity for they confer passive protection on mice not only against infection with Type III pneumococci but against Types II and VIII organisms as well.

Although cellobiuronic acid antiserum causes agglutination and *Quellung* only with the Type III Pneumococcus it must be borne in mind that protection tests are far more subtle than are these gross qualitative phenomena and that protection can be demonstrated with amounts of antibody which cannot be detected with other techniques. Furthermore, it has been proven that cellobiuronic acid is a constituent of the Type VIII pneumococcus polysaccharide. For these reasons, therefore, it is not out of the question that the protection which cellobiuronic acid antiserum affords mice against infection with Type VIII pneumococci can be attributed to the identity in structure of a portion of the polysaccharide molecule. The striking results obtained with Type II Pneumococcus cannot be explained until a more comprehensive understanding of the uronic acid constituent of the capsular polysaccharide of this microorganism is had. The results of the foregoing experiments indicate the importance of ascertaining the exact constitution of the specific polysaccharides of encapsulated pathogens, for it is only through such knowledge that the enigma of their specificities will be fully explained.

SUMMARY

1. An artificial antigen containing the azobenzyl glycoside of cellobiuronic acid gives rise in rabbits to antibodies which: (*a*) give the Neufeld reaction and agglutinate Type III pneumococci, (*b*) confer passive protection on mice against infection with Types II, III, and VIII pneumococci.

2. Rabbits immunized with the artificial cellobiuronic acid antigen acquire active resistance to infection with virulent Type III pneumococci.

3. The antibodies evoked by an antigen containing the azobenzyl glycoside of cellobiose exhibit none of these phenomena.

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