IMMUNOLOGICAL RELATIONSHIPS OF CELL CONSTITUENTS OF PNEUMOCOCCUS.

SECOND PAPER.

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(Received for publication, June 8, 1925.)

In the course of studies carried on in this laboratory during the past 3 years on the chemical and immunological properties of the cell constituents of Pneumococcus, certain facts have been acquired which indicate a significant relationship between the chemical constitution and biological specificity of the bacterial cell.[1,2] It seems desirable at this time to bring together in summary form these facts in order to relate them to the problem as a whole.

In the work thus far, two of the cellular constituents of Pneumococcus have been studied. One of these is the substance precipitated from solutions of Pneumococcus by dilute acetic acid, which, although comprising a mixture of proteins, may for the purposes of the present discussion be referred to as bacterial nucleoprotein. The other constituent, non-protein in character, is the so called soluble specific substance, which is now considered to be a carbohydrate of the polysaccharide type. While it is realized that these two substances, important as they are, do not comprise the whole antigenic mosaic of the cell, they are emphasized at this time because they happen to be the first chosen for investigation, and the only ones thus far isolated.

Studies of the antigenic and serological properties of these two chemically diverse substances as they exist together in the intact cell and free in a dissociated state, have yielded certain facts which contribute to a better understanding of the differential specificity of the

[1] For the first paper see Avery, O. T., and Heidelberger, M., *J. Exp. Med.*, 1923, xxxviii, 81.

[2] *Cf.* in part the two preceding papers, also the chemical studies from this laboratory referred to in these.

various types of pneumococci. For not only do these cellular compounds belong to two wholly different classes of chemical substances namely protein and carbohydrate, but the carbohydrate constituent of each of the three fixed types of pneumococci has been found to be a chemically different polysaccharide. These substances possess the unique distinction of reacting specifically with antibacterial serum of the homologous type, although in the free state, dissociated from the cell, they are devoid of the power of inciting the formation of antibodies upon injection into animals. The protein, on the other hand, is antigenic; the serum of an animal immunized with this substance reacts with protein derived from any type of Pneumococcus. It at once becomes obvious that as these two components exist in the cell, they enter into an antigenic complex which has different immunological properties from those exhibited by either substance alone. The serological differentiation of the various types of pneumococci is dependent, therefore, not only on the chemical and antigenic differences in these component substances, but is also intimately related to the structural character and morphological integrity of the bacterial cell.

That these principles, so strikingly exemplified in the immunological reactions of Pneumococcus, are of more general biological significance is illustrated in the somewhat analogous relationships existing within other groups of bacteria and of yeasts. The work of Zinsser, Mueller, and their associates (1) indicates that from a number of microorganisms, such as staphylococci, meningococci, and Friedländer, typhoid, and tubercle bacilli, a non-protein residue substance may be extracted which bears a definite relation to the specific character of the bacteria-cell. Smith and Reagh (2) in 1903 described differences in the serol logical behavior of motile and non-motile bacilli of the hog-cholera group. Recently Orcutt has shown that these differences are dependent upon two separate agglutinogens corresponding to the flagellar and somatic substances of the cell; the presence of both of these factors in the motile forms and the absence of the flagellar antigen in non-motile types account for the variations observed in the immunological behavior of these bacilli. Felix (3) and others have found that the differences in agglutination of the O and H forms of *proteus* bacillus are related to the occurrence in these organisms of one or both of

two separate substances, involving the endo- and ectoplasm of the cell. These analogies while close are not absolute; the flagellar substance of motile bacilli is in itself antigenic, while the soluble specific substance of Pneumococcus is a non-antigenic carbohydrate. The significant fact, however, is that in these widely divergent groups of microorganisms two distinct cellular substances are determinative factors in bacterial specificity.

Structure of the Cell.—Before considering in detail the immunological characters of these two cellular constituents of Pneumococcus it may add to the clearness of the discussion to picture the form or pattern of the cell as it relates to the disposition of these substances. For undoubtedly cell configuration reflects in some measure the ease with which this organism participates in immunity reactions and the avidity with which it interacts with antibody. Many of these reactions are presumably surface phenomena, and the nature of the reactive material at the periphery of the cell may determine the readiness of response and even the specificity of reaction. Pneumococcus is an encapsulated organism, and there are grounds for the belief that the ectoplasmic layer of the cell is composed of carbohydrate material which is identical in all its biological characters with the type-specific substance of Pneumococcus. On the other hand, the endoplasm, or somatic substance, consists largely of protein which, as previously pointed out, is species- and not type-specific. This protein is possessed in common by all pneumococci while the carbohydrate is chemically distinct and serologically specific for each of the three fixed types. The cell, therefore, may be conceived of as so constituted that there is disposed at its periphery a highly reactive substance upon which type specificity depends. The structure and, as will be pointed out later, the morphological integrity of the cell are determinative factors in bacterial specificity.

Chemistry of the Soluble Specific Substance.—The following data briefly summarize our knowledge of the soluble specific substance of the three fixed types of Pneumococcus at the stage of purification so far attained (4).

The soluble specific substance of Type II pneumococcus appears to be a *weakly* acidic, nitrogen-free polysaccharide made up chiefly of glucose units. Its specific optical rotation is about *plus* 74°. It

reacts at a dilution of 1:5,000,000 with antibacterial serum of Type II pneumococcus, and does not react with Type I or Type III antisera.

The soluble specific substance of Type III pneumococcus, while also apparently a nitrogen-free polysaccharide, differs from the Type II derivative in many particulars. It rotates the plane of polarized light about 33° to the *left.* It is a *strong* acid and is made up of units of glucose and either glucuronic acid or some derivative of this acid. It also separates in insoluble form from solutions strongly acidified with hydrochloric acid. In as high a dilution as 1:6,000,000 it still reacts with Type III antipneumococcus serum.

TABLE I.

Chemical Characteristics of the Soluble Specific Substances of Types I, II, and III Pneumococcus.

Type.	Optical rotation.	C	H	N	Acid equivalent.	Reducing sugars on hydrolysis.		Highest dilution giving precipitate with homologous immune serum.
						per cent		
I	+300°	43.3*	5.8	5.0†		28	(Galacturonic acid.) (Amino sugar derivative.)	1:6,000,000
II	+74°	45.8	6.4	0.0	1250	70	Glucose.	1:5,000,000
III	−33°	42.6	5.6	0.0	340	75	Glucose (glucuronic acid).	1:6,000,000

* Theory for $(C_6H_{10}O_5)_x$: C = 44.4 per cent; H = 6.2 per cent.

† Amino N: 2.5 per cent.

The Type I soluble specific substance (4), on the other hand, while also polysaccharide in nature, differs from the other two type-specific substances in containing nitrogen as an apparently essential component. In spite of a nitrogen content of 5.0 per cent the substance gives none of the usual protein color tests. One-half of the nitrogen is liberated when the substance is treated with nitrous acid. Reducing sugars appear at the same time and the specific reaction vanishes, and since the carbon and hydrogen content are close to the theoretical values for polysaccharides it appears likely that a nitrogenous sugar derivative is involved. It is a strong acid and a weak base, and is very sparingly soluble in water at the isoelectric point. Its specific optical rotation is +300°, and on oxidation with nitric acid it yields mucic

acid. In the specific precipitin reaction with homologous antipneumococcus serum it can be detected in dilutions as great as 1:6,000,000.

In Table I are summarized the available data concerning the chemical differences in the polysaccharide derivatives of pneumococci of Types I, II, and III.

Although the specific polysaccharide by itself evokes no antibodies upon injection into rabbits it is specifically reactive with antibody induced by immunization with intact cells. The fact that this specifically reactive carbohydrate is non-antigenic when dissociated from the other cellular constituents and is capable of inciting antibody formation only in the form in which it is present in the intact cell, forces the conclusion that in the latter instance it exists not merely as free carbohydrate but also in combination with some other substance which confers upon it specific antigenic properties. Immunization with intact bacteria containing this carbohydrate complex elicits antibodies which not only agglutinate the formed cells but precipitate solutions of the carbohydrate isolated from pneumococci of the homologous type. How the specific polysaccharide is combined in the cell, whether with protein or some other constituent is not yet clear, but it is evident that the compound thus formed is the dominant and essential antigen of the cell, and the one responsible for type specificity.

The immunological relationships of the protein and carbohydrate fractions of the cell are graphically presented in Fig. 1, in which *S* represents the specific soluble substance (carbohydrate) and *P* the protein of the Pneumococcus. The symbols used are in no sense to be construed as interpretive of the mechanism involved, but serve simply to visualize the interaction between these cell constituents and their respective antibodies. The reactions illustrated may be briefly summarized as follows:

A (*Fig. 1, A*).—Immunization with intact cells of a given type of Pneumococcus gives rise in the serum to the presence of antibodies which are type-specific; such sera specifically agglutinate the homologous type of Pneumococcus, protect mice against virulent organisms of the same type, and precipitate solutions of the corresponding purified carbohydrate (Table II). While this type-specific carbohydrate antibody is provoked only in response to the antigenic stimulus of the whole cell, a serum containing this antibody alone without a trace of

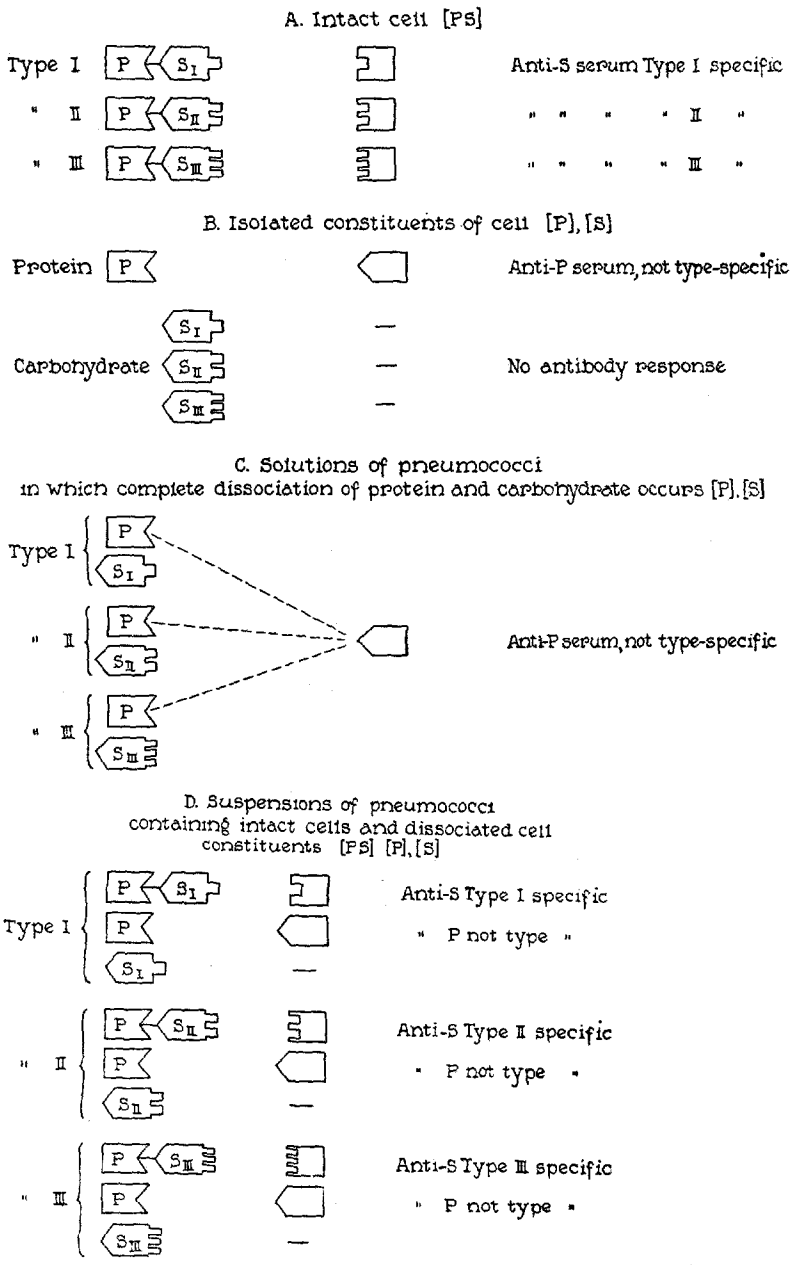

FIG. 1. Immunological relationships of protein and soluble specific substance (carbohydrate) of Pneumococcus.

protein antibody is rarely obtained for reasons which will be discussed later. Whether singly or in conjunction with other antibodies this specific carbohydrate antibody has been observed only under conditions in which the intact bacterial cell has been employed for immunization.

B (*Fig. 1, B*).—The specific polysaccharide (*S*) when isolated from the cell is incapable of inciting antibody formation upon injection into

TABLE II.

Pneumococcus and cell constituents		Antibodies demonstrable in serum						
Material used for immunization	Effective antigen	Agglutinins	Precipitins		Complement fixation		Specificity	
			S	P	S*	P	Type	Species
Intact cells $[SP]_{\dagger}$	$[SP]_{\dagger}$	+	+	−	+	−	+	−
Carbohydrate $S_{\ddagger}$	None	−	−	−	−	−	−	−
Protein $P_{\ddagger}$	P	−	−	+	−	+	−	+
Solutions, extracts containing free S and free P	P	−	−	+	−	+	−	+
Suspension of intact cells and dissociated cell constituents [SP], free S, free P	[SP], P	+	+	+	+	+	+	+

$_{\dagger}$[SP] = Carbohydrate and protein combined antigen of cell

$_{\dagger}$S = Free carbohydrate, the soluble specific substance of cell

$_{\ddagger}$P = Free protein of cell

*Free S, as antigen, does not fix complement with immune horse serum; is active with immune rabbit serum[3]

animals. The isolated protein (*P*), on the other hand, is antigenic and gives rise to an immune serum which reacts with pneumococcus protein regardless of the type from which it is derived. Antiprotein sera do not agglutinate type-specific strains of pneumococci and do not precipitate solutions of the soluble specific substance (*S*) (Table II).

C (*Fig. 1, C*).—Solutions and extracts of pneumococci behave antigenically precisely as do solutions of pneumococcus protein; the disso-

[3] *Cf.* Vollmond, E. (in press).

ciation of the antigenic complex which occurs whenever the cell is dissolved results in the liberation of free carbohydrate (*S*) and free protein (*P*) in solution. In such solutions and extracts the only constituent which functions as antigen is the protein, for free *S* is non-antigenic. The sera of animals immunized with solutions of pneumococci, in which complete dissociation of these cell constituents has taken place, contain only antibodies reactive with the protein. Such sera exhibit the same reactions as do those prepared by immunization with protein alone (Table II).

It is evident from these facts that morphological dissolution of pneumococci is accompanied by antigenic dissociation, for sera prepared from filtered solutions of disintegrated cells free of formed elements fail to exhibit any of the dominant type-specific properties which characterize sera obtained by immunization with whole bacteria (compare *A* and *C*, Fig. 1). Morphological integrity of the bacterial cell, therefore, is requisite for the expression of its full antigenic power.

D (*Fig. 1, D*).—It becomes obvious, therefore, that the character of the antibody response is determined by the nature of the cell material used for immunization. The injection of suspensions of pneumococci into animals induces the formation of antibodies against *S* alone or against both *S* and *P* separately, depending upon whether or not these suspensions contain only intact cells or a mixture of both intact and dissolved cell bodies. Since pneumococci readily undergo autolysis and dissolution, suspensions and indeed cultures of these organisms almost invariably contain not only formed elements, but also more or less of dissociated cell constituents in solution. The predominance of the former in such suspensions stimulates the production of the type specific *S* antibodies, while the occurrence in these same suspensions of dissociated cell protein provokes the formation of the species-specific protein antibodies. The more common result of immunization with cell suspensions is the occurrence in the serum of both the carbohydrate and protein antibodies. The former invariably predominate, the latter are present in varying concentration depending upon the amount of cell dissolution which has taken place in the material previous to or after injection into the animal body. Therefore, use of suspensions of pneumococci containing both intact cells and the soluble products of cell disintegration yields on immunization not only type-

specific antibodies but antibodies reacting with the protein substance which is common to all pneumococci. It is the presence of this protein antibody with its broader zone of activity which is responsible for the confusing cross-immunity reactions occasionally encountered in supposedly type-specific sera. That these two antibodies are separate and distinct is shown by absorption tests; the antiprotein reacting bodies in such sera can be removed by absorption with the protein of a heterologous type without diminishing the titer of specific agglutinins for the homologous culture or the precipitins for the specific polysaccharide of the corresponding type.

The relationship of the carbohydrate constituent of Pneumococcus to type specificity, and the establishment of its identity with the soluble specific substance previously discovered (5) in culture fluids and in the urine and blood of patients suffering from pneumococcus pneumonia are facts indicative of the biological importance of this substance both to the bacterial and animal organisms.

The elaboration of this specific carbohydrate is a specialized function most active in pneumococci rapidly multiplying in the animal body or in suitable culture media. The stimulus to the full expression of this function is found under circumstances providing optimal growth conditions. Under these conditions pneumococci exhibit maximal capsular development, exalted virulence, and distinct type specificity. Conversely, when optimal conditions are lacking the organisms may become degraded, lacking capsules, of little or no virulence, and devoid of type distinction. Without further supporting evidence it would of course be hazardous to venture the assertion that all three of these characters are necessarily and causally related to the *S*-producing function of the cell. However, the earlier work of Stryker (6) and the more recent studies of Griffith (7), Reimann (8), and Amoss (9), as well as our own observations, indicate that under certain cultural environments pneumococci lose their type specificity and become avirulent. Moreover, these changes are now known to be accompanied by a loss of the capacity to elaborate this soluble specific substance.

In 1917 Cole (10) observed that infected exudates and sera containing these soluble reactive substances of pneumococci possessed the property of neutralizing pneumococcus antibodies, and pointed out the

significance of this fact in relation to the therapeutic administration of immune serum. Moreover, the work of Sia (11) on the effect of this carbohydrate substance on the growth of pneumococci in normal serum-leucocyte mixtures indicates that the addition of this substance in very small amounts exerts a definite and specific effect in annulling the inhibitory action of the sera of naturally resistant animals.

If final proof be brought for the conception that the capsular zone of the organism is largely composed of this carbohydrate substance, is part of the defense mechanism of the cell, and is the site of its initial contact with antibody, then these soluble bacterial polysaccharides acquire new significance not only in the serological reactions of the cell, but in the actual processes of infection and immunity in the host.

SUMMARY.

In this paper the general immunological significance of the intact pneumococcus cell and of its protein and carbohydrate components is discussed.

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