STUDIES ON MENINGOCOCCUS INFECTION

IV. The Antigenic Complex of the Meningococcus—Group-Specific Carbohydrate and Protein Fractions

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In a previous paper (1) it has been pointed out that other investigators have demonstrated the existence of at least two group-specific, serologically active substances, one a protein (2) and the other a polysaccharide (2, 3), which can be obtained from extracts of meningococci. The present paper deals with two similar if not identical group-specific fractions which have been isolated from broth cultures of meningococci of various types after the method described in that paper (1). Only the serological reactions and certain other properties of these two fractions will be described here; the detailed chemical study of these substances, together with that of the type-specific fractions, will be the subject of an additional communication (4).

The Group-Specific Carbohydrate

That fraction of the autolysate which can be precipitated by eight volumes of 95 per cent ethyl alcohol, and which appears as a white powder after partial purification has been obtained from Types I, II and III and proves to be identical in each of the types and similar to, though not identical with, a fraction obtained by much the same technique from other microorganisms. It resembles in most of its properties the polysaccharide of the meningococcus described by Zozaya (2, 3), but differs from this in showing cross-reactions with antipneumococcal serum. This interreaction between antimeningococcal and antipneumococcal sera and the corresponding polysaccharide fractions serves to throw much light on a fact which has been noted not infrequently in the literature; namely, the presence of precipitinogens reacting with polyvalent antimeningococcal serum in

body fluids of patients suffering from pneumococcal infections, especially in the cerebrospinal fluid of patients with pneumococcal meningitis (see Vincent and Bellot (5) and Dopter (6)). Boor and Miller (7), working with a non-protein fraction of the gonococcus, found a relationship between gonococcus, meningococcus and pneumococcus similar to that described below for the meningococcus fraction. In accordance with the term adopted for the similar fractions obtained from other microorganisms, this group-specific fraction from the meningococcus has been termed the "C substance."

As has been stated in the preceding paper of this series (1), the C substance may be rendered more pure by repeated alcohol precipitation. After the first two or three reprecipitations all of the precipitate is readily redissolved in distilled water, giving a clear, colorless solution.[1] A standard dilution of 1/100 may be kept in the ice box for a period of months without serious loss of serological activity, but the C substance keeps even better in the form of a dry powder which may be dissolved and used as required.

This carbohydrate fraction gives an abundant precipitate with some but not all samples of polyvalent antimeningococcal serum,[2] though it gives only a very slight precipitate with the monovalent rabbit sera prepared with freshly isolated strains in the manner described elsewhere (8).

Experiment 1.—A specimen of C substance obtained from Strain 23 (Type I) was set up, in a dilution of 1/100, against the four monovalent sera and a polyvalent antimeningococcal horse serum which previous experience had shown to possess a high precipitin titre (see Table I).

It will be seen that while the precipitate is heavy with the polyvalent serum, it is negligible with the monovalent sera prepared from

[1] Although, in the description of the isolation of this C substance from the crude broth (1), the statement is made that on alcohol precipitation a heavy white precipitate settles out, this is not invariably the case and, especially when the broth has been concentrated, in its place there may appear a copious deposit of a brownish gum. The isolation of the C substance from this gum is a matter of much difficulty and recent work indicates that this group-specific fraction can be isolated more readily from extracts of organisms grown on solid media.

[2] For the specimens of polyvalent antimeningococcal horse serum the author is indebted to Dr. J. Zozaya and the New York State Department of Health.

fresh strains. It may be pointed out that the Type IV serum, in which the precipitate is heaviest, is prepared from a stock strain, no fresh strain being available.

The C substance from each of the three types reacts equally well with the polyvalent serum and absorption of the polyvalent serum with the C substance of one type removes the precipitins for the C substance not only of that type but of all the types.

Experiment 2.—Specimens of C substance from each of Type I, II and III strains in dilutions of 1/100 were set up against (1) unabsorbed polyvalent serum

TABLE I

I	II	III	IV	Polyvalent	
No. 578	No. 592[3]	No. 266	No. 577	No. 3	
0	0	0	0	+^{r}	Immediate } Ring test
∓^{r}	∓^{r}	∓^{r}	±^{r}	+++^{p}	1 hr. } Ring test
∓	∓	∓	±	+++^{d}	2 hrs.
∓	∓	∓	±	++++^{d}	Ice box

r = ring.
p = granular precipitate.
d = disc.
∓, ±, +, ++, +++, ++++ = increasing amounts of precipitate.

No. 3, and (2) polyvalent serum No. 3 absorbed twice with C substance from Strain 19, Type II. The ice box readings are given in Table II.

The serological identity of the polysaccharide from the three different types is clearly shown by the results of this absorption test.

The C fraction is precipitated by other sera than polyvalent antimeningococcal serum. Antigonococcal serum and antipneumococcal Type III serum gave good reactions, while antipneumococcal Type II serum and one sample of antistreptococcal serum[3] gave slighter

[3] For this and other antistreptococcal sera, the author is indebted to Dr. Rebecca Lancefield.

reactions. Antipneumococcal Type I sera, other specimens of antistreptococcal serum and sera prepared against the Gram-negative bacilli gave no reaction. The strong reaction with Type III antipneumococcal serum and the weak or negative reaction with Type II and Type I sera are in keeping with the fact that Type III serum always contains plentiful precipitins for the pneumococcus C substance, while sera prepared against Types I and II contain little. Table III shows the results of precipitin experiments between antimeningococcal polyvalent serum, antigonococcal serum, antipneumococcal sera and the active specimen of streptococcal serum and a specimen of meningococcus C substance (from Strain 23), diluted 1/100 and 1/1,000.

TABLE II

Type of C substance	I	II	III
Strain No.	23	19	302
Unabsorbed polyvalent serum	++++[d]	++++[d]	++++[d]
Polyvalent serum absorbed with Type II C	∓	∓	∓

TABLE III

Serum	Meningococcus		Gonococcus		Pneumococcus I		Pneumococcus II		Pneumococcus III		Streptococcus	
Dilutions of C substance	10^{-1}	10^{-2}	10^{-1}	10^{-2}	10^{-1}	10^{-2}	10^{-1}	10^{-2}	10^{-1}	10^{-2}	10^{-1}	10^{-2}
23 C	++++[d]	+++[p]	+++[p]	+[p]	0	0	±	0	++[p]	0	±[p]	±

Conversely, polyvalent antimeningococcal serum and, to a lesser extent, antigonococcal serum will give a precipitate with the C substance of other microorganisms.

Experiment 3.—A specimen of pneumococcus C substance, highly purified and diluted 1/100, was obtained through the kindness of Dr. Forrest Kendall. This was set up against polyvalent antimeningococcal, antigonococcal and antipneumococcal Type III sera (Table IV).

The precipitates formed from the interaction of the antimeningococcal and antipneumococcal sera and the pneumococcus C substance were marked. That from the antigonococcal serum was less.

When these sera are absorbed with meningococcus C substance all

the precipitins for the homologous C substance are removed, while those for the heterologous C substances are decreased but not altogether removed. Similarly, absorption of the sera with pneumococcus C substance removes the homologous precipitins completely but causes only a partial decrease in the heterologous precipitins.

Experiment 4.—Polyvalent antimeningococcal serum and Type III antipneumococcal serum were absorbed with dry meningococcus C substance (Strain 19,

TABLE IV

Serum	Meningococcus	Gonococcus	Pneumococcus III
	No. 2	No. 011105-C	No. 81B-1
Pneumococcus C 1/1,000	$+++^{p}$	$+$	$++^{p}$

TABLE V

Serum		Polysaccharide	
		Meningococcus C	Pneumococcus C
Meningococcus serum	Unabsorbed	$++++^{d}$	$+++_{p}$
	Absorbed with meningococcus C substance	$\mp$	$++^{p}$
	Absorbed with pneumococcus C substance	$+++^{d}$	0
Pneumococcus serum	Unabsorbed	$++^{p}$	$++_{p}$
	Absorbed with meningococcus C substance	0	$+\pm_{p}$
	Absorbed with pneumococcus C substance	$\pm^{p}$	0

Type II), 10 mg. to 0.5 cc. of serum, for 2½ hours at 37°C. and overnight in the ice box. At the same time, similar quantities of the two sera were absorbed with 0.8 mg. of pure pneumococcus C substance (obtained through the courtesy of Dr. Walther Goebel). These absorbed sera were tested against meningococcus and pneumococcus C substance diluted 1/100 (Table V).

These results would appear to indicate that the C substances obtained from different organisms, while very similar, are not identical chemically or immunologically. This differentiation of the two poly-

saccharides by means of the absorption test calls to mind a similar differentiation made by Avery, Heidelberger and Goebel (9) between the specific polysaccharides of Friedländer E and Pneumococcus Type II which had been found to resemble one another so closely. The case of the meningococcus and pneumococcus C substances, however, would seem to differ from that of the Friedländer E and Pneumococcus Type II specific substances in one particular. Avery, Heidelberger and Goebel found that absorption of Friedländer serum with the homologous organism removed all the precipitins and not merely those for the Friedländer E specific substance, but that absorption of this serum with the Pneumococcus Type II organisms removed only the precipitins for the Pneumococcus Type II specific substances. In the same way, homologous absorption of the pneumococcus serum removed all precipitins, while absorption with Friedländer organisms removed only the precipitins for Friedländer specific substance. In the case of the C substance of the meningococcus and pneumococcus, absorption of either serum with either C substance removes only the homologous precipitins and merely decreases, but does not remove, those for the polysaccharide other than that used in absorption. Since it was thought that the difference might be in the fact that in the latter case C substance alone was used for absorption while Avery, Heidelberger and Goebel had used whole organisms, the experiment with the meningococcus and pneumococcus sera was repeated using whole organisms for absorption.

Experiment 5.—Polyvalent antimeningococcal serum and Type III antipneumococcal serum were absorbed with whole meningococci. The growth used was a 16 hour culture of a freshly isolated Type II strain grown on blood agar plates, washed off in saline and centrifuged down, the serum being then added to the packed organisms which were mixed in with steady shaking of the tube. The absorbed sera and the same sera without absorption were now set up against solutions of meningococcus and pneumococcus C substance, both at 1/100. Table VI shows the results.

It will be seen that, in complete agreement with the work of Avery, Heidelberger and Goebel, the whole meningococci absorb from the antimeningococcal serum all of the precipitins, not only for the homologous but also for the heterologous C substance. On the other hand, absorption of the antipneumococcal serum with meningococci removes

only the precipitins for the homologous C substance and leaves the pneumococcus C precipitins unaffected.

The C substance of the meningococcus will give a precipitin reaction in dilutions of one part in a million. This is a higher titre than is usually obtained with any of the type-specific substances. This may in part be due to the fact that the polyvalent antimeningococcal horse serum used in testing the C substance has of itself a higher titre than the monovalent rabbit sera used with the type-specific substances.

TABLE VI

Serum		Polysaccharide	
		Meningococcus C	Pneumococcus C
Meningococcus serum	Unabsorbed	+++[p]	+++[p]
	Absorbed with meningococcus whole organisms	∓	0
Pneumococcus serum	Unabsorbed	+±[p]	++[p]
	Absorbed with meningococcus whole organisms	0	++[p]

TABLE VII

Dilutions of C substance....	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	Dilutions of serum....	1/1	1/2	1/4	1/8	1/16	1/32
Polyvalent serum 1/1	++++[d]	++++[d]	++[p]	+	∓	0	C substance 1/1,000	++++[d]	+++[d]	++[p]	++[p]	++[p]	+[p]

Experiment 6.—A specimen of meningococcus C substance (from Strain 23, Type I), purified by six reprecipitations with eight volumes of 95 per cent ethyl alcohol, was diluted from 1/100 to 1/10,000,000 and set up against undiluted polyvalent serum No. 3. Dilutions of the serum from 1/1 to 1/32 were set up against the C substance diluted 1/1,000 (Table VII).

It will be noticed also that C substance diluted 1/1,000 will give a precipitate in serum dilutions out as far as 1/32.

Chemical Analysis.—Purified specimens of C substance giving precipitin reactions out to 1/1,000,000 show a strong Molisch reaction at 1/100, a weak one at 1/1,000 and are negative at 1/10,000. Neither

a positive biuret nor a positive trichloracetic acid test for protein can be obtained at 1/100.

The P Substance

This fraction—the P substance—, which is precipitated from the autolysate by means of 10 per cent acetic acid, appears as a greyish white flocculent precipitate. Unlike the C fraction and the type-specific substances which pack easily on centrifugation, the P substance is difficult to remove completely from the supernatant fluid and does not form a tight pack even after prolonged centrifugation. To some extent this appears to be due to the presence of mucoproteins which do not precipitate readily. The precipitate may be dissolved in N/100 Na_2CO_3 and then treated by repeated acetic acid precipitation and sodium carbonate resolution. After every precipitation there is a residue which will not pass back into solution. This residue decreases but slightly with further purifications, and must probably be regarded as protein denatured during the process of purification. It is fully realized that the sample of so called nucleoprotein with which one finishes may well be somewhat different from that occurring *in vivo*, or even on first precipitation, but one can conclude that whatever changes have taken place have been unable to alter to any great extent the reactive properties of the substance which still precipitates in a characteristic manner with the appropriate serum. It may also be stated at this point that it is doubtful whether any of the protein material obtained in this, the accepted method, from microorganisms consists of nucleoprotein, despite the common appellation of nucleoprotein applied to this material in the literature. Certainly, very little can be regarded as nucleoprotein and the bulk consists of other conjugated proteins which preliminary work suggests can be separated one from another by various manipulations. Only very small amounts of carbohydrate are present even in the first crude specimens of P substance. A constant feature, however, is the presence of type-specific substance in the first crude specimens and persisting even for the first few purifications. This fact is clearly demonstrated when crude specimens of P substance from the different types are tested with monovalent and polyvalent sera (Table VIII).

It will be noted that the crude P fraction, when tested with monova-

lent serum, reacts only with sera homologous to the type from which it is derived. The type-specific substance to which this reaction is due can be removed by purification, giving a solution which still reacts strongly with the polyvalent antimeningococcal serum but no longer with any of the monovalent sera. Where the type-specific fraction is present in sufficiently large amounts it can be recovered by precipitation with two volumes of ethyl alcohol and the specimen thus obtained is much purer than that obtained by the usual method, since it contains only undemonstrable amounts of C substance, if any at all.

TABLE VIII

Serum	I	II	III	IV	Polyvalent No. 3
No. 428 Type I P^2	+	0	+	0	++++^{P}
No. 31 Type II P^2	0	±	0	0	++++^{P}
No. 302 Type III P^2	++^{P}	0	+++^{P}	0	++++^{P}

TABLE IX

Serum	Meningococcal		Gonococcal		Pneumococcal	
Dilutions of P substance	10^{-2}	10^{-3}	10^{-2}	10^{-3}	10^{-2}	10^{-3}
	++++^{P}	++^{P}	++^{P}	±	++^{P}	0

Like the C substance, the protein fraction reacts with both antigonococcal and Type III antipneumococcal sera (Table IX), but not with any of the sera tried; *viz*,, antistreptococcal sera and sera prepared against the Gram-negative bacilli. This is in keeping with the work of Boor and Miller (7) who showed that the nucleoprotein which they obtained from the gonococcus was likewise not species-specific.

The P fraction will give a precipitin reaction in a dilution of 1/100,000 but not in 1/1,000,000, which is lower than that obtained with the C substance when the same polyvalent antimeningococcal serum is used.

Experiment 7.—A specimen of meningococcus P substance (from Strain 428, Type I), precipitated by three reprecipitations with 10 per cent acetic acid, was set up in dilutions of from 1/100 to 1/10,000,000 against polyvalent serum (No.

3) undiluted. Dilutions of the serum up to 1/32 were set up against a 1/1,000 solution of P substance (Table X).

P substance at 1/1,000 reacts with a serum dilution of 1/16 but not one of 1/32.

Chemical Analysis.—Purified specimens of P substance giving precipitin reactions out to 1/100,000 give a negative Molisch reaction at 1/100. Both biuret test and precipitate test with 20 per cent trichloracetic acid are positive at 1/10,000 dilution, but not at 1/100,000.

All of the specimens of P substance which have been tested have proved to be highly toxic for rabbits and, to a much less degree, for mice. The toxicity of various preparations varies to some degree, but the majority have a strength such that 1 to 3 mg. injected intravenously will kill a 2,000 gm. rabbit within 6 hours. The toxic factor is relatively stable and the suspension of P substance can be stored in

TABLE X

Dilutions of P substance....	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	Dilutions of serum.....	1/1	1/2	1/4	1/8	1/16	1/32
Polyvalent serum 1/1	++++P	++P	±	∓	0	0	Protein 1/1,000	++P	++P	+	±	∓	0

the ice box over a period of weeks without showing any great loss in toxicity. Specimens of P substances, besides being toxic, are strongly antigenic and will evoke a serum which, in addition to having a high precipitin titre for the meningococcus P substance, will protect against many lethal doses of the toxic factor contained in the P substance. It must be stated here that it is by no means certain at the moment that the P substance and the toxic factor are one and the same thing. Investigations are at present under way concerning this particular point.

DISCUSSION

Three substances, which react in a specific manner with appropriate sera, have been isolated from the meningococcus. Of these substances, the first or type-specific substance has been described in a previous paper (1), while the other two, which are group-specific, have been described above.

The method of isolation used for these substances has been chiefly that of prolonged growth of the organisms in fluid media with autolysis occurring *pari passu* with this growth. It is realized that such prolonged cultivation in fluid media must give rise to many substances which are products of the bacterial metabolism or are formed as a result of the interaction of the organism and its pabulum, the surrounding medium. In such a case, doubt may exist as to whether the serologically active substances are preformed in the organism or are not rather the result of interaction of meningococci and media. In order to surmount this objection, other methods of obtaining the serologically active substances have been used. The organisms have been grown on solid media for short periods of time, *i.e.* under 20 hours, and then washed off. The bacterial growth obtained in this manner has been extracted with saline, weak alkalies or acids, and the extracts so obtained have been subjected to methods much the same as those applied to the broth cultures. By such means it has proved possible to isolate three serologically active substances which now behave in like manner to those obtained from the broth. Zozaya and Wood (2) and Zozaya (3) used similar methods for the isolation of group-specific protein and polysaccharides of the meningococcus. The method which calls for the use of solid media has not been adopted as a routine because of several complicating factors. Foremost of these is the influence of the agar. As Sordelli and Mayer (10) and others have pointed out, saline solution or any other solvent brought into contact with agar, dissolves up some fraction present in the agar, which will now give a precipitin reaction with sera produced by the inoculation of vaccines prepared from bacteria grown on agar plates. Since all sera used in the present work are prepared from agar-grown vaccines, it is clear that complications will arise in the use of organisms grown on media with this basis for the preparation of the serologically active substances. Apart from this is the fact that growth in broth is a far less laborious method of preparing the crude substances and one which gives a far greater yield.

The fact that sera containing antibodies for these chemical substances isolated from the broth cultures can be obtained by the inoculation of young and presumably intact cultures of meningococci would lead to the assumption that such chemical substances, or others per-

haps more complicated but containing the essential radicals, must be present in the intact organism and not be a product merely of metabolic activity. This assumption is further supported by the fact that similar substances may be obtained from young organisms not allowed to autolyze but killed and extracted immediately, as has been pointed out above.

It is believed that these fractions bear a relation to certain of the facts recognized in connection with the meningococcus itself and with the reaction of the host to invasion with this organism. It would seem clear that the type specificity of the meningococcus, which by the use of a modified agglutination technique (8) seems to be a definite and distinct entity, depends, at least in part, on the presence within the organism of the appropriate member of the type-specific substances described and, moreover, that the specificity of agglutination and other serological reactions depends on the interaction of that substance and the corresponding antibody in the serum.

The clear-cut type specificity of the serological reactions is best seen with sera prepared against freshly isolated strains of meningococci. Sera prepared from stock strains tend to give group-specific as well as type-specific reactions, especially when lower serum dilutions are used. This would appear to be due to the fact that, as has been shown by Petrie (11) and in this laboratory, strains of meningococci when repeatedly subcultured on artificial media lose much of the type-specific substance which characterized them in the freshly isolated state; indeed, several of the differences to be noted between stock and fresh strains may be due to this fact. When such stock strains are used in the production of serum, the type-specific antibodies are formed only slowly and prolonged immunization is required to obtain a serum of sufficient antibody content. The serum produced by such a period of immunization is one of broad non-specificity giving cross-reactions, at least in lower dilutions. It would seem that the animal, while reacting to the type-specific substance, has formed antibodies also against the group polysaccharide and protein (C and P) fractions. The presence of antibodies for these group substances in the serum will account for the cross-reactions. The animal appears to react more readily to the type-specific than to the group substances, for it is a fact that antibodies against the former appear sooner in the serum than those

against the latter. Why this should be is at present not clear. It may be that the type-specific substance lies near the surface of the organism, but it should be emphasized that the meningococcus apparently does not possess a capsular structure of type-specific substance such as is presumed for the pneumococcus. It is nevertheless a fact that when fresh strains with plentiful type-specific substance are used, sera with a sufficiently high titre of type-specific antibodies but with low titre of group-specific antibodies can be obtained promptly. On the other hand, to obtain a serum with group-specific antibodies, using whole organisms, it is necessary to immunize over a period of many months (12).

The antigenic structure of the organism probably accounts for certain other peculiarities of the agglutination reaction. It has been shown (8) that agglutination at 37°C. is more satisfactory than that at 56°C., owing to the absence of cross-reactions. It may be said in parenthesis that this applies only to the use of sera prepared with fresh strains (anti-S sera), since sera prepared with stock strains seem to be too low in type-specific antibodies to react at the lower temperature. Using anti-S sera in low dilutions, the reaction is rapid and clear-cut at 37°C.; at 56°C., however, cross-agglutination tends to obscure the results and the suspended organisms appear to lose their type specificity. It can be shown that warm saline, in which meningococci have been suspended for some hours and then centrifuged out, contains type-specific substance. One may therefore suppose that agglutination at 56°C. is unsuitable because at this temperature the type-specific substances diffuse out readily into the surrounding fluid and the group substances, either by being exposed or by becoming predominant, react with their appropriate antibodies to produce non-specific agglutination.

Both type-specific and group substances have been demonstrated in the body fluids of those suffering from meningococcus infections. They can be shown to exist in the spinal fluid in cases of meningitis due to this organism, where they give a precipitin reaction with anti-S monovalent sera (13). As might be expected, the strength of precipitin reaction obtained is directly dependent on the numbers of meningococci in the spinal theca which have undergone autolysis, and thus, more indirectly, on the total number of infecting organisms. Where

this is very low, the precipitin titre is also very low or absent; moreover, treatment with antimeningococcal serum tends to diminish or remove entirely the precipitinogens, so that a reaction is frequently no longer to be obtained once therapeutic treatment has been commenced.

SUMMARY

Two group-specific substances, one a polysaccharide and the other a protein, have been isolated from the meningococcus and their serological properties are described. A discussion of these substances, together with the type-specific fraction, is given, and a relationship of these substances to certain serological phenomena is suggested.

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