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New Classification of *Neisseria meningitidis* by Means of Bactericidal Reactions

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A bactericidal assay is described which allows identification of distinct serotypes within a serogroup of *Neisseria meningitidis*. Antisera produced in rabbits against seven group C strains by two intravenous inoculations of live organisms were found to contain two types of bactericidal antibodies. One, directed against the group-specific polysaccharide, caused various degrees of killing of all strains. Absorption of this antibody by purified group C polysaccharide revealed the presence of the second bactericidal antibody. This antibody was directed against antigenically distinct factors associated with serotype specificity. Extensive cross-absorption yielded antisera with activity directed against four separate factors. The presence of a factor in a strain was indicated by its susceptibility to killing by antisera containing antibody to that factor. A serotype was defined by the particular combination of factors. Six different serotypes, containing one or two factors, were identified among 16 group C strains examined.

Sixty years ago, Dopter (5) first classified *Neisseria meningitidis* on the basis of the bacterial agglutination reaction. Since then, many workers have refined and expanded the serogrouping of the meningococcus (1, 3, 6, 8, 13, 16) so that today strains can be separated into at least nine groups: A, B, C, D, X, Y, Z, 29E, and 135.

More detailed serological classification of meningococcal strains into subgroups or types has not been described. That such a system of typing, analagous to that for salmonellae or streptococci, may be possible was first indicated by Roberts (15). He was able to distinguish two antigenically distinct strains of group B meningococci by using a bactericidal technique. Goldschneider et al. (7), also using a bactericidal assay, subsequently reported that there is complex antigenic diversity within group C strains.

We now report the development and application of a bactericidal test which allows distinct typing of group C strains. Preliminary observations on the uses of this typing scheme in the study of the epidemiology of meningococcal disease are presented and discussed.

MATERIALS AND METHODS

Culture methods. All strains of meningococci were from the Walter Reed Army Institute of Research collection, stored in the lyophilized state within three to five passages from initial isolation. On the basis of antigenic differences suggested by the work of Goldschneider et al. (7), seven group C strains were initially chosen for study: 60E, 1381, 1714, 913, 126E, 1185, and 321. After overnight growth on BYE agar (BBL) at 37 C in a CO_2 incubator, a large loopful of organisms was inoculated into 25 ml of Mueller-Hinton broth (Difco) in a 250-ml nephelometry flask and incubated aerobically at 37 C in a rotary-shaker water bath. Organisms were harvested during the early log phase of growth when the optical density, measured in a Coleman Junior spectrophotometer at 600 nm, was 0.1. This value was attained within 45 to 90 min after inoculation of the flask and was found to correspond to a concentration of approximately 3×10^8 organisms per ml.

Preparation of antiserum. Antiserum to whole organisms was prepared in New Zealand rabbits weighing 4 to 5 lb (1.8 to 2.3 kg) by the intravenous administration of 1.0 ml of a broth culture containing approximately 3×10^8 viable organisms. Two such injections were given at 6- to 8-week intervals. The rabbits were exsanguinated 2 to 3 weeks after the second injection. Serum was obtained by centrifugation of clotted blood, sterilized by filtration through a 0.45-nm membrane filter (Millipore Corp., Bedford, Mass.), and stored in 1- to 5-ml samples at −70 C. Before use, all antisera were inactivated at 56 C for 30 min.

Antiserum to group C meningococcal polysaccharide [lot 9, produced by the Squibb Medical Research Institute by the method of Gotschlich et al. (9) and referred to hereafter as C polysaccharide] was prepared in New Zealand rabbits by the method of Plescia et al. (14). The polysaccharide was first complexed with methylated bovine serum albumin

(Worthington Biochemical Corp.) and then mixed with complete Freund's adjuvant (Difco). A total dose of 1 mg of polysaccharide was injected subcutaneously into the footpad in a course of three injections at weekly intervals. The rabbits were exsanguinated 10 to 14 days after the third dose, and the serum was obtained and stored as described above.

Bactericidal assay. The bactericidal test employed in these studies is a modification of the procedure described by Roberts (15). The composition of the reaction mixtures is described below. Diluent (GGS) was 0.1% sterile gelatin (Difco) in Gey's Balanced Salt Solution (Microbiological Associates). As a source of complement, 10% normal rabbit serum (NRS) was included in all of the reaction mixtures. Rabbits were selected as complement sources only after their sera had been shown to lack bactericidal activity against the strains being tested. The serum was obtained as described above and stored in 1-ml samples at −70 C. It was thawed immediately before use. The test strain of meningococci was grown as described above. Because there was no significant difference in the bactericidal activity of sera against organisms over the range 10^3 to 10^8 organisms per ml, the broth culture containing 3×10^8 organisms per ml was diluted with GSS to obtain an inoculum of 3×10^4 organisms per ml. With this inoculum, samples of the reaction mixture could be plated directly to determine the colony count without need for further dilutions.

Reaction mixtures were set up in sterile screw-cap tubes (16 by 75 mm) as follows. Complement control consisted of 0.8 ml of GGS, 0.1 ml of NRS, and 0.1 ml of meningococci. Test mixture consisted of 0.7 ml of GGS, 0.1 ml of NRS, 0.1 ml of antiserum, and 0.1 ml of meningococci. The reaction mixtures were incubated aerobically at 37 C in a rotary-shaker water bath. Because it was found that killing was essentially complete within 10 to 15 min, viable colony counts were performed at 0 and 30 min by plating duplicate 0.1-ml samples of each reaction mixture on Mueller-Hinton agar.

For analysis of results, bactericidal activity is expressed in terms of the per cent killing in the test mixture compared to the complement control. Greater than 90% killing is indicated in the tables by +, 20 to 90% killing is indicated by ±, and less than 20% killing is indicated by 0. Less than 20% killing was not considered significant because duplicate tests showed variations of 10 to 15%.

Absorption of antiserum with whole organisms. Antiserum was absorbed with whole organisms by a modification of the method of Hollis et al. (10). A loopful (3 mm) of organisms from an overnight growth on BYE agar was added to approximately 3 ml of heat-inactivated antiserum. The mixture was incubated for 1 hr at 37 C and then for 18 to 24 hr at 4 C. The organisms were removed by centrifugation followed by filtration through a 0.45-nm membrane filter (Millipore Corp.), and the process was repeated for a total of three absorptions. Antisera treated in an identical fashion, but without the addition of organisms, showed no change in bactericidal activity.

Absorption of antiserum with sheep erythrocytes sensitized with C-polysaccharide. Sheep erythrocytes were fixed with pyruvic aldehyde by the method of Ling (11) and sensitized with C polysaccharide by the method of Gotschlich et al. (9). Antiserum was then mixed with an equal volume of a 50% suspension of sensitized, fixed red blood cells and incubated for 1 hr at 37 C and then overnight at 4 C. The cells were removed by centrifugation and the serum was sterilized by filtration.

Inhibition of bactericidal activity by C-polysaccharide. Serial twofold dilutions of C polysaccharide in GSS were prepared in the range between 3.9 and 2,000 μg/ml. A sample of each dilution of polysaccharide was incubated for 30 min at 37 C with an equal volume of a 1:16 dilution of antiserum. The bactericidal activity present in each inhibited antiserum was then determined in the usual bactericidal test.

A second type of inhibition experiment was performed in which a constant amount of polysaccharide solution containing 2,000 μg/ml was added to an equal volume of a series of twofold dilutions of antiserum. After incubation of the mixtures for 30 min at 37 C, the bactericidal activity was assessed.

RESULTS

Efficacy of the immunization schedule. As reported by Roberts (15), it was found that a single intravenous injection of approximately 3×10^8 organisms induced bactericidal activity in the sera of all rabbits tested 5 to 6 weeks after immunization. The titers giving greater than 90% killing after a single injection were 1:40 to 1:80. A booster given 6 to 8 weeks after the primary immunization increased these titers to 1:320 to 1:640.

With fixed sheep red blood cells sensitized with C polysaccharide (9), transitory low titers (1:32 or less) of hemagglutinating antibody were detected within 1 week after immunization. The immunization schedule did not result in the development of agglutinating antibody or in precipitating antibody detectable by agar-gel diffusion.

Preliminary antigenic differentiation by two group C strains. Antisera to strains 60E and 1381 were compared for bactericidal activity against homologous and heterologous strains, both before and after absorption with whole organisms and with C polysaccharide-sensitized red cells (SRBC-C_s). Each strain was killed by both homologous and heterologous unabsorbed antiserum (Table 1). Similar killing of heterologous strains by unabsorbed antiserum was found with almost all combinations of antisera and strains. Absorption of antiserum by its homologous strain resulted in removal of all bactericidal activity.

The effect of absorption with heterologous whole organisms or with SRBC-C_s was essentially

TABLE 1. *Bactericidal activity of antisera after absorptions with heterologous strains or with red blood cells sensitized with C-polysaccharide*

Strain	Killing by indicated antiserum[a]							
	Anti-60E/0	Anti-60E/60E	Anti-60E/1381	Anti-60E/C_R	Anti-1381/0	Anti-1381/1381	Anti-1381/60E	Anti-1381/C_R
60E	+[b]	0	+	+	+	0	0	0
1381	+	0	0	0	+	0	+	+

[a] In this and subsequent tables, absorbed antisera are indicated as follows: anti-60E/0 is unabsorbed anti-60E; anti-60E/1381 is anti-60E absorbed by strain 1381; and anti-60E/C_R is anti-60E absorbed by C-polysaccharide-sensitized red cells.

[b] Symbols: +, greater than 90% killing; 0, less than 20% killing.

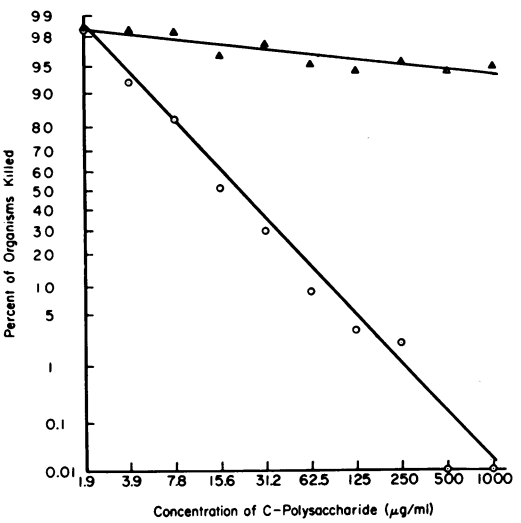

FIG. 1. *Bactericidal activity of anti-60E serum against strain 60E (△) and strain 1318 (○) at different concentrations of C-polysaccharide. Normal deviate ordinate scale; logarithmic abscissa scale.*

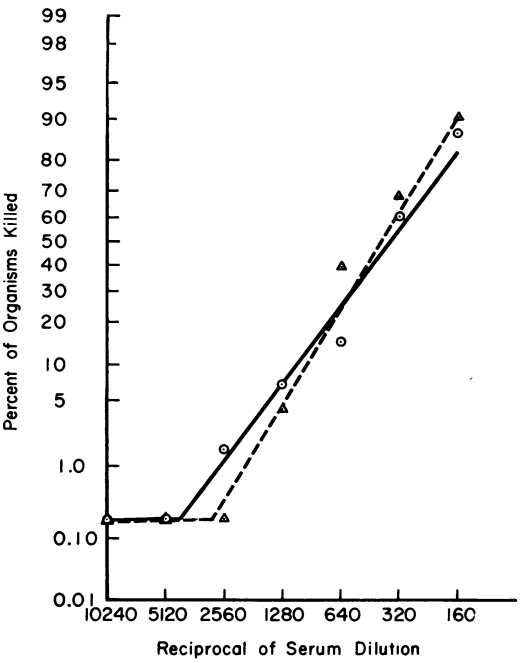

FIG. 2. *Bactericidal activity of anti-60E serum against strain 60E: comparison of effects of absorption with strain 1381 (○) and inhibition with 1,000 μg of C-polysaccharide per ml (△). Normal deviate ordinate scale; logarithmic abscissa scale.*

TABLE 2. *Bactericidal activity of anti-60E/1381 serum and of anti-1381/60E serum against five group C strains*

Strain	Killing by indicated antiserum	
	Anti-60E/1381	Anti-1381/60E
913	0[a]	+
1714	0	+
321	+	0
1185	0	0
126E	0	0

[a] Symbols: +, greater than 90% killing; 0, less than 20% killing.

the same. Absorption of anti-60E serum with strain 1381 or with SRBC-C_s removed all activity against the heterologous strain 1381 without affecting the killing of strain 60E. Conversely, the absorption of anti-1381 serum with 60E or with SRBC-C_s removed activity against strain 60E without reducing the killing of strain 1381. These absorption results suggest that strains 60E and 1381 possess an antigen or antigens that are distinct. Moreover, it appears that the killing of heterologous strains by unabsorbed antiserum is mediated by antibody directed against the common group-specific polysaccharide. Antiserum to purified C polysaccharide behaved like unabsorbed antiserum to whole organisms in that it killed all group C strains.

Confirmation of this view was obtained by inhibition of bactericidal activity with the C polysaccharide. Samples of a 1:16 dilution of anti-60E serum were mixed with serial twofold dilutions of C polysaccharide over a range of final concentrations of 1.9 to 1,000 μg/ml. The bactericidal activity of the inhibited antisera was then tested against strains 60E and 1381. The results were

analyzed graphically by the probit analysis method (12). As seen in Fig. 1, there was still over 90% killing of strain 60E by anti-60E serum even in the presence of 1,000 μg of C polysaccharide per ml; on the other hand, the killing of strain 1381 was reduced to 50% by as little as 15.6 μg of polysaccharide per ml and was completely inhibited by 500 μg/ml.

That absorption with a heterologous strain resulted in the removal of antibody to the C polysaccharide was examined in another way. The killing of strain 60E by serial dilutions of anti-60E serum absorbed with strain 1381 was compared to that by serial dilutions of anti-60E mixed with 1,000 μg of C polysaccharide per ml (Fig. 2). The curves of the two antisera preparations are practically identical, with no significant difference in the slope or in the 50% end point. The identity of the two lines is a further confirmation that absorption with a heterologous strain and inhibition by C polysaccharide have the same effect, namely, the removal or inhibition of cross-reacting antibody directed against the group C-specific polysaccharide.

The results of these preliminary experiments suggested that the bactericidal reaction might provide the means of distinguishing between antigenic types of group C meningococci, especially after removal of the common antibody to the C polysaccharide from strain-specific antisera.

Development of factor sera for typing group C meningococci. In the preceding section, the results of experiments were presented which demonstrated that strain 60E could be distinguished serologically from strain 1381. The antigen or antigens distinct to strain 60E are designated factor I; the antigen or antigens distinct to strain 1381 are called factor II. Anti-60E/1381 serum contains antibody to factor I (anti-I); anti-1381/60E serum contains antibody to factor II (anti-II).

The bactericidal activities of anti-I sera (anti-60E/1381) and anti-II sera (anti-1381/60E) were determined against five additional group C strains (Table 2). It is apparent that, like strain 1381, strains 913 and 1714 contain factor II but not factor I. After being absorbed with strain 60E, the antisera to strains 913 and 1714 had bactericidal activity identical to that of anti-1381/60E. Moreover, reciprocal cross-absorptions between the three strains removed all bactericidal activity. Therefore, strains 1381, 913, and 1714 are antigenically identical as revealed by the bactericidal reaction and contain only factor II.

Returning to Table 2, we observe that strain 126E was not killed by either anti-I or anti-II sera. Indeed, strain 126E was not killed by absorbed antisera to any of the other six strains, i.e., anti-60E/1381, anti-1381/60E, anti-1714/60E, anti-913/60E, anti-321/60E, or anti-1185/60E. The only antiserum which killed strain 126E was the homologous anti-126E serum. Absorption of anti-126E with any of the other strains did not remove its bactericidal activity. Therefore, strain 126E contains a different factor, factor III, and anti-126E/60E serum has anti-III antibody. Anti-III serum did not kill any strain except 126E.

Of the two remaining strains in Table 2, strain 321 was killed by anti-I serum but not by anti-II serum, whereas strain 1185 was killed by neither. Reciprocal cross-absorptions of antisera to strains 60E and 321 were performed as indicated in Table 3. Absorption of anti-60E with strain 321 removed bactericidal activity against both strains, confirming the presence of factor I in 60E and 321. Absorption of anti-321 with strain 60E removed factor I antibody and it no longer killed strain 60E. However, antibody to an additional factor must have been present in anti-321/60E since it killed strain 321. This new factor is designated factor IV, and anti-321/60E contains anti-IV antibody. Absorption of both anti-60E and anti-321 sera with strain 1714 containing factor II had no effect on the killing by either serum of strains 60E and 321, thus confirming the absence of factor II from both strains.

Strain 1185 was not killed by antisera to factors

TABLE 3. *Bactericidal activity of anti-60E and of anti-321 after reciprocal cross-absorptions by strains 321, 60E, and 1714*

Strain	Killing by indicated antiserum	
	Anti-60E/321	Anti-321/60E
60E	0[a]	0
321	0	+

[a] Symbols: +, greater than 90% killing; 0, less than 20% killing.

TABLE 4. *Bactericidal activity of anti-321 and anti-1185 sera after absorptions with strains 60E, 1381, 1714, 1185, and 321*

Strain	Killing by indicated antiserum					
	Anti-321/60E	Anti-321/1714	Anti-321/1185	Anti-1185/60E	Anti-1185/1381	Anti-1185/321
321	+[a]	+	+	+	+	0
1185	+	+	0	+	+	0
60E	0	+	±	0	0	0

[a] Symbols: +, greater than 90% killings; ±, 20 to 90% killing; 0, less than 20% killing.

I, II, or III. The effect of anti-321/60E serum on strain 1185 is shown in Table 4, along with that of certain other absorptions of anti-321 and anti-1185 sera. The killing of 321 and 1185 by both anti-321/60E and anti-1185/60E sera indicates that both strains have factor IV. However, unlike strain 321, 1185 does not contain factor I. The effect of reciprocal cross-absorption of antisera to 321 and 1185 is also shown in Table 4. Neither anti-321/1185 nor 1185/321 serum killed strain 1185, indicating the removal of anti-IV antibody from both antisera. However, anti-321/1185 serum did kill both 321 and 60E, confirming the persistence of anti-I antibody despite the absorption with strain 1185.

Having identified four factors among the seven strains selected for study, we next prepared factor sera such that each contained antibody to only one factor. These sera were: anti-I, anti-60E/1381; anti-II, anti-138/60E; anti-III, anti-126E/60E; anti-IV, anti-1185/60E or anti-321/60E. The bactericidal activity of the four factor sera against the seven strains are summarized in Table 5, along with the activity against nine other group C strains. This table indicates the antigen composition of each strain in terms of the four factors identified to date.

Thus far, in studying a total of 16 group C strains, only 4 factors have been identified. Nine strains have a single factor and seven have two factors.

TABLE 5. *Bactericidal activity of factor sera against 16 group C strains*

Strain	Killing by indicated factor antiserum			
	Anti-I	Anti-II	Anti-III	Anti-IV
59E	+[a]	0	0	0
60E	+	0	0	0
476	+	0	0	0
1381	0	+	0	0
913	0	+	0	0
1714	0	+	0	0
142E	0	+	0	0
126E	0	0	+	0
1185	0	0	0	+
321	+	0	0	+
384	+	0	0	+
743	+	0	0	+
981	0	+	0	±
1401	0	+	0	+
1661	0	+	0	+
1583	0	+	0	+

[a] Symbols: +, greater than 90% killing; ±, 20 to 90% killing; 0, less than 20% killing.

DISCUSSION

The identification of serogroups by the bacterial agglutination reaction has long been the only method of serological classification of *N. meningitis* strains. This paper describes the development of a bactericidal assay which allows identification of distinct serotypes within a serogroup. During the course of this work, the findings of Roberts (15) and Goldschneider et al. (7) that distinct antigenic differences exist between strains within a serogroup have been confirmed.

The essential difficulty in recognizing strain differences within a serogroup lies in the fact that there are two distinct kinds of bactericidal antibodies present in rabbit anti-meningococcal serum (7). One antibody is directed against the group-specific polysaccharide and results in varying degrees of killing of all group C strains. This reaction leads to the masking of the killing by the type-specific antibodies. Removal of the anti-C polysaccharide antibody is, therefore, essential for the demonstration of antigenic differences between strains. It has been shown that its removal can be accomplished not only by absorption or inhibition with purified C polysaccharide but also by absorption with live intact group C organisms of a heterologous type. With such absorbed sera, one is able to identify distinct serotypes within what has generally been considered a uniform serogroup.

By further cross-absorptions between strains, four antigenically distinct factors among seven group C strains were found. What appeared to be monospecific factor sera were prepared by appropriate cross-absorptions. Their ability to kill or not kill a given strain was taken to indicate the presence or absence of that factor. Different combinations of these factors defined the serotype of the strain. The examination of 16 group C strains with the four-factor sera revealed the existence of six serotypes (Table 6). Additional strains are under study to test for the existence of other factors.

The use of the agglutination reaction to serogroup meningococci has yielded several generalizations about the epidemiology of meningococcal

TABLE 6. *Distribution of factors among 16 group C strains*

Factors	Strains
I	59E, 60E, 476
II	1381, 913, 1714, 142E
III	126E
IV	1185
I + IV	321, 384, 743
II + IV	981, 1401, 1661, 1583

disease. A definite correlation can be made between epidemic disease and group A strains (2). Although there is widespread nasopharyngeal carriage of all serogroups except group A, most endemic disease in the United States is caused by groups B and C. It has recently been shown that the ratio of attack rate to carrier rate varies among the serogroups (4).

However, there are many epidemiological problems that have resisted solution by use of the agglutination reaction. Little is known about the dynamics of transmission of strains within closed populations. There are many gaps in what is known about the carrier state. There is no definite information about differences in virulence, invasiveness, or transmission potential of different strains. These are but a few of the problems the solution of which may be aided by the identification of serotypes by means of the bactericidal factor test. Such a system may prove to be analagous to the Kauffmann-White classification of *Salmonella* and hopefully will be as beneficial in the study of the epidemiology of the meningococcus.

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