Classification of *Neisseria meningitidis* Group B into Distinct Serotypes

II. Extraction of Type-Specific Antigens for Serotyping by Precipitin Techniques

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Over 10 distinct serotypes of group B *Neisseria meningitidis* have been found to date by using a sensitive microbactericidal assay developed by the authors. The serotype antigens have now been extracted by hot acid or saline extraction procedures. It was found that these extracted serotype antigens may be used in a simple capillary precipitin method. This method uses unadsorbed, undiluted rabbit antisera. In the capillary precipitin method a 3+ to 4+ reaction was considered significant. The microbactericidal assay and capillary precipitin methods for serotyping group B meningococci show excellent agreement. Group B meningococci were also serotyped by an agar gel double diffusion technique. The latter technique conserves reagents and has the further advantage that it does not show the minor cross-reactions observed in the capillary precipitin method. Thus two simple, reproducible methods for serological typing of group B meningococci have been developed. These methods developed for the serological typing of group B meningococci will aid in epidemiological studies of meningococcal disease. They may also be of value for selecting suitable strains for vaccine production.

In 1945 Branham stated that group B *Neisseria meningitidis* was a very heterogeneous group and required a thorough serological investigation (1). Roberts in 1967 (14) observed that antisera prepared against two group B meningococcal strains failed to show reciprocal opsonic and bactericidal antibody. This led him to suggest that bactericidal antibodies against group B meningococci were type-specific rather than group-specific. Based upon these observations, we have recently developed a sensitive microbactericidal assay. By using this assay we have to date been able to subdivide the group B meningococci into 10 distinct serotypes (3).

The present study will show that these serotypes have specific antigens associated with them which can be extracted by hot acid or saline. These extracted serotype antigens enabled the development of a simple capillary precipitin test for serotyping group B meningococci.

**MATERIALS AND METHODS**

**Bacteriological techniques.** The source of strains, culture techniques, and maintenance of the organisms were as described previously (3).

**Antisera.** Immune rabbit sera that were prepared for our microbactericidal assay were used in these experiments. The immunization procedure was as described previously (3). Hyperimmune antisera were prepared by repeating the complete immunization schedule after a 3-week rest period.

**Extraction of type-specific antigens.** Different group B meningococcal strains were grown in 150 ml of Trypticase soy broth (TSB; BBL) overnight at 37 C on a gyratory shaker (New Brunswick Scientific Co.). The cells were harvested by centrifugation at 2 C, 6,000 X g and washed once with normal saline. The packed cells were resuspended in 0.7 ml of 0.0167 N HCl prepared in normal saline. Care was taken that the pH of the initial mixture of cells and acid was not below 4.5. The cells were then heated in covered test tubes for 10 min in a 100 C water bath. The tubes were quickly cooled. One drop of 0.05% phenol red was added, and the mixture was then neutralized to pH 7.0 to 7.2 by dropwise addition of 0.0167 N NaOH. A clear supernatant extract obtained by centrifugation at 2 C and 6,000 X g was stored at 4 C until used. This supernatant fraction will be referred to as the acid extract.

Another satisfactory extraction procedure was also developed. Two milliliters of normal saline was added to the washed packed cells obtained from overnight growth in 150 ml of TSB. The resuspended cells were heated in covered test tubes for 30 min in a 100 C
water bath. After immediate cooling, the cell extract was centrifuged as before to obtain a clear supernatant fluid which will be referred to as the saline extract.

**Capillary precipitin technique.** The capillary precipitin technique used here is a modification of the method used for M-typing group A hemolytic streptococci (16). Small-diameter capillary tubes were used (0.5 to 0.9-mm inner diameter by 75 mm long). Undesorbed, undiluted antisera was first drawn into the capillary and then an equal volume of undiluted extract. The precipitin reactions were read with aid of a X3.5 hand lens after incubation at 37 C for 2 hr and then read again after overnight refrigeration at 4 C. The reactions were read ± 4+ based upon the criteria used for M-typing group A hemolytic streptococci (16). A significant precipitin reaction was considered to be 3+ or 4+.

**Agar gel double diffusion.** The method used was the Ouchterlony technique (10). Six glass microscope slides were held in an LKB plastic tray (Gelman). A 16-ml amount of melted Noble agar in normal saline containing 0.01% (w/v) thimerosal (Merthiolate) as preservative was added to the tray. After solidification, two circular sets of agar wells with either six or eight peripheral wells were cut on each glass slide with an LKB gel punch (Gelman no. 6866A). Undiluted antisera and undiluted extracts were used in the diffusion tests. The precipitin reactions were read after 1, 2, and 3 days at room temperature in a moist chamber.

**Microbactericidal assay.** This technique was described in our previous report (3).

**Adsorption of antisera.** Adsorbed antisera were prepared for some experiments. The method of adsorption was as previously described (3).

**RESULTS**

**Parameters for optimal antigen extraction.** In preparing acid extracts for the precipitin tests, the acid strength and amount of organisms were of major importance. A study was made to determine the optimal pH range for acid extraction by using 0.7 ml of 0.2 to 0.01 N HCl in saline added to equal portions of washed cells. The initial pH of the acid plus cell mixture was determined with a Beckman pH meter with microelectrodes. It was found that for acid extraction the pH could be controlled by the normality of the HCl used. Extraction below pH 4 (in reaction mixture) resulted in apparent absence of the serotype antigen in the extract. The optimal pH range for acid extraction was between pH 4.5 and 6.0.

When saline extracts were made, the initial pH of the reaction mixture after the addition of the saline to the cells was approximately 6.0.

The amount of cells in the extraction mixture was critical. An extract prepared from the overnight growth in 50 ml of TSB often failed to give precipitates. An extract prepared from a larger amount of cells resulting from overnight growth in 150 ml of TSB was usually quite satisfactory.

An extract may also be obtained by autolysis, but this extract shows numerous nonspecific precipitin reactions. In addition, we have found that the clear supernatant fluid obtained after acid or saline extraction must be removed from the cells as soon as possible to prevent similar nonspecific reactions.

The neutralized extracts when sealed and stored at 4 C were stable for approximately 3 months. After this time the precipitin activity of the extract decreases noticeably.

**Specificity of the capillary precipitin reaction.** Having developed the extraction procedure, we then determined whether or not the precipitin reactions were type-specific by using the acid extracts. Unadsorbed undiluted antisera were tested against a series of different group B strains which had been previously serotyped by the microbactericidal assay (3). It can be seen in Table 1 that, except for serotype 1 antisera, each of the type-specific antisera reacted strongly (3+ to 4+) only with an acid extract of a single strain. As determined by the bactericidal assay, strain M978 had serotype antigens 1 and 8, and in the precipitin test an extract of M978 reacted strongly with both anti-1 and anti-8 sera.

In precipitin reactions, only a 3+ or a 4+ reaction was considered significant. Some unadsorbed antisera such as anti-5, anti-8, and anti-11 (Table 1) reacted only with a single extract. However, since unadsorbed antisera were used throughout, it was not unexpected when minor cross-reactions of 1+ were found.

We have previously reported (3) several group B strains which have the serotype antigen 2. These type 2-associated strains may also carry other serotype antigens, i.e., they are multiple serotypes. When capillary precipitin tests were performed on extracts of these type 2 strains using either homologous or heterologous type 2-associated antisera (Table 2), strong precipitin reactions were observed. The single case wherein a strong precipitin reaction was not shown was anti-B16B6 tested against M988 extracts. However, anti-B16B6 was also not bactericidal for M988. Since anti-M988 showed 4+ precipitin activity against B16B6 extracts, the reason for this nonreciprocal activity remains unclear. Despite this inconsistency, it is clear from Table 1 that type 2 antisera react strongly with extracts of type 2-associated strains.

**Correlation of bactericidal and precipitin tests.** From the preceding studies, the precipitin reaction using acid extracts appeared to be type-specific. Saline extracts contain serotype antigens comparable to those of acid extracts (C. E. Frasch, Ph.D., thesis, Univ. of Minnesota, Min-
neapolis, 1972). The observed precipitin reactions were next correlated with the microbactericidal assay. Table 3 shows a representative comparison of precipitin and bactericidal activities. For most antisera tested, bactericidal activity against a strain correlated with a strong precipitin reaction against an extract of that strain. The minor precipitin reactions of 1+ to 2+ observed with unadsorbed serotype antisera, such as with serotype 6 antisera (Tables 1 and 3), seldom correlated with bactericidal data.

In most cases, there was excellent agreement between a strongly positive precipitin reaction and the presence of bactericidal activity. However, some antisera gave good type-specific precipitin reactions but failed to show any bactericidal activity (Table 4).

It can be seen in Table 4 that strains M460, M996, and M988 are identifiable as type 2-associated strains because of the bactericidal activity of type 2 antisera against these strains and by the strong precipitin reactions of their extracts in type 2 antiserum. Yet, antisera prepared against these strains by the same immunization method failed to show any homologous bactericidal activity, but these antisera did contain excellent type-specific precipitin antibodies. Anti-

### Table 1. Precipitin activity of serotype antisera against group B strain extracts

<table>
<thead>
<tr>
<th>Serotype antiserum</th>
<th>Activity against strain extracts</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>M266</td>
</tr>
<tr>
<td>1</td>
<td>3+</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
</tr>
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<td>0</td>
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<tr>
<td>8</td>
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</tr>
<tr>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>11</td>
<td></td>
</tr>
</tbody>
</table>

* Strength of precipitin reaction from 1+ (weak) to 4+ (strong).

### Table 2. Precipitin activity of type 2-associated strain antisera against different type 2-associated strain extracts

<table>
<thead>
<tr>
<th>Serotype antiserum</th>
<th>Type-specific antibodies present</th>
<th>Activity against extracts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>B16B6</td>
</tr>
<tr>
<td>Anti-B16B6</td>
<td>2a</td>
<td>4+</td>
</tr>
<tr>
<td>Anti-M986</td>
<td>2, 7</td>
<td>4+</td>
</tr>
<tr>
<td>Anti-M988</td>
<td>2, 7</td>
<td>4+</td>
</tr>
<tr>
<td>Anti-M1011</td>
<td>2, 10</td>
<td>4+</td>
</tr>
<tr>
<td>Anti-M616</td>
<td>2, 7, 10</td>
<td>4+</td>
</tr>
</tbody>
</table>

* As determined previously by microbactericidal assay.

### Table 3. Correlation of bactericidal and precipitin activity

<table>
<thead>
<tr>
<th>Antiserum against type</th>
<th>Activity against strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MGC-15</td>
</tr>
<tr>
<td></td>
<td>Pptn</td>
</tr>
<tr>
<td>1</td>
<td>4+</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
</tr>
</tbody>
</table>

* Pptn = precipitin; strength of precipitin reaction from 1+ (weak) to 4+ (strong).

M136 serum (serotype 11) also showed the presence of strong precipitins in the absence of bactericidal activity.

The presence of strong type-specific precipitin activity in the absence of bactericidal antibody may indicate that in some cases the precipitating antibody and bactericidal antibody may be...
directed against different antigenic determinants of the type-specific antigen or antigens. The antisera prepared in two different rabbits against M988, a type 2 strain, possessed good type-specific precipitin activity but failed to kill the homologous strain, although M988 was killed by other type 2 antisera. Subsequently, a third rabbit produced antisera against M988 having excellent precipitating antibodies and a homologous bactericidal titer of 1:1,280. It is suggested that the type-specific antigenic determinants induce precipitating antibodies and that these antibodies may also be bactericidal if directed against a suitable cell surface antigenic determinant.

Another strain, M996, was used to immunize rabbits, producing antisera with strong type-specific precipitins yet no detectable bactericidal antibody (Table 4). These rabbits were then given an additional 5-week immunization with strain M996. The resulting hyperimmune antisera contained no demonstrable bactericidal antibody in all dilutions tested yet had quite strong precipitating antibodies against acid and saline extracts of all group B strains. Thus, prolonged immunization had induced precipitating antibodies against an antigen common to various group B strains. This demonstrates the undesirability of prolonged immunization for the production of type-specific antisera.

Adsortion of precipitin and bactericidal activity. Thus far a strong correlation has been shown between the bactericidal and capillary precipitin tests. It was assumed that the capillary precipitin and bactericidal antibodies were generally directed against the same antigenic determinants. This was further investigated by adsorbing different strain antisera with homologous and heterologous strains before testing for bactericidal and capillary precipitin activity (Table 5). We found that

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>M460</th>
<th>M996</th>
<th>M988</th>
<th>M136</th>
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<tbody>
<tr>
<td></td>
<td>Pptn&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Cidal&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Pptn</td>
<td>Cidal</td>
</tr>
<tr>
<td>Anti-M460</td>
<td>4+</td>
<td>N</td>
<td>4+</td>
<td>N</td>
</tr>
<tr>
<td>Anti-M996</td>
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<td>4+</td>
<td>N</td>
<td>4+</td>
<td>N</td>
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<tr>
<td>Anti-M136</td>
<td>0</td>
<td>N</td>
<td>0</td>
<td>N</td>
</tr>
<tr>
<td>Type 2</td>
<td>4+</td>
<td>P</td>
<td>4+</td>
<td>P</td>
</tr>
</tbody>
</table>

* Pptn = precipitin; strength of precipitin reaction from 1+ (weak) to 4+ (strong).
* Cidal = bactericidal; P, bactericidal activity present at titer of 1:40 or greater; N, no bactericidal activity at titer of 1:20.

This result from Table 4 demonstrates that adsorption with the homologous strain for 1 hr at 37°C followed by 1 hr at 4°C removed all precipitin and bactericidal activity from the homologous antisera. Adsorption with heterologous strains did not alter either precipitin or bactericidal activity. This was interpreted to mean that the type-specific antigens detected by bactericidal antibodies were extractable by acid or saline and demonstrable by capillary precipitins. That the same antigens were involved in both systems has been further shown by the ability of acid or saline extracts to inhibit only the homologous bactericidal reaction.
Agar gel double diffusion studies. Acid extracts were examined by the agar gel double diffusion technique. We found that many extracts of group B strains formed a single precipitin band with their homologous antisera (Fig. 1) but showed no precipitin lines with heterologous antisera. The type-specific precipitin band generally formed near the antigen well.

We then examined the type 2-associated strains containing multiple serotype antigens to determine whether they would show single or multiple precipitin bands. In Fig. 2 an anti-2,7 serum in the center well was tested against extracts of eight different strains. Three of these extracts contained type 2-associated antigens. We had expected to see separate precipitin lines for each serotype antigen. However, as seen in Fig. 2, only a single specific precipitin line was observed. This was an indication that antigen 2 was a single antigen with multiple antigenic determinants. This was strongly supported by the finding of lines of partial identity between some type 2-associated strains known to differ in at least one antigenic determinant (Fig. 3). A line of partial identity may be seen between the anti-2,7 and anti-2 sera reacting with the multiple serotype extract containing antigens 2 and 7 in center well.

In addition to the characteristic type-specific precipitin lines which formed near the antigen well, there may be a second sharp precipitin line which forms about halfway between the antigen and antibody wells and appears at the same time as the type-specific precipitin line. The antigen represented by this line is not type-specific but common to many group B strains (Fig. 4).

DISCUSSION

Roberts (14) made the initial observation that antisera prepared against two group B meningococcal strains did not show reciprocal opsonic or bactericidal activity. Based upon this observation, we set out to develop a sensitive serological
method that would enable us to distinguish distinct serological types within group B meningococci. After we had developed a sensitive microbactericidal assay (3), this procedure was used to provisionally establish 10 distinct serological types.

It is likely that all of the major serological groups of *N. meningitidis* will prove to be divisible into a series of subgroups or types. Evidence that group C may be divisible into antigenically distinct types was shown by Gold and Wyle (5). In addition, groups B and C have recently been subdivided on the basis of their sensitivity to bacteriocins (2). In the case of group B, there appears to be no relationship between our bactericidal serotypes and bacteriocin sensitivity.

Investigators have usually had difficulty demonstrating precipitins against the group-specific polysaccharide antigens of group A and C meningococci (6, 11, 12, 15). However, the group B polysaccharide is not well demonstrable by precipitin tests (7, 11, 12). We find no precipitin lines by gel diffusion traceable to the group-specific polysaccharide of group B. This is advantageous to our precipitin test since the group-specific antigen of group B does not interfere with serotyping group B meningococci.

Precipitins have been demonstrated against protein antigens of group B (8, 9, 13, 15). Menzel and Rake (9, 13) isolated proteins from autolysates of group B that appeared to be group-specific and others that appeared to be species-specific. These protein antigens precipitated strongly in group B antiserum. The kappa substance of Menzel and Rake (9) was a carbohydrate containing polypeptide which precipitated strongly only in group B antiserum. Kappa substance, we believe, is distinct from our serotype antigens based upon our recent immunochromatographic studies (C. E. Frasch and S. S. Chapman, Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 89, 1972). These immunochromatographic studies of group B meningococcal serotype antigens are presently being prepared for publication in this laboratory.

Jyssum (8) has also isolated different protein and polysaccharide fractions from group B meningococci by potassium thiocyanate digestion, and only the protein fractions gave demonstrable immunoprecipitates by agar gel diffusion.

For the serological typing of group B meningococci we have now developed a simple capillary precipitin method. We found that the type-specific antigens could be extracted by hot acid or saline and that these antigens could then be detected by their formation of immunoprecipitates with type-specific antisera. Thus by a simple extraction procedure and an easily performed precipitin test it is now possible to distinguish between strains of group B meningococci.

The capillary precipitin results closely agree with the bactericidal typing of group B meningococcal strains. Adsorption of a type-specific antiserum with the homologous strain simultaneously removed all bactericidal and precipitin activity. An extract prepared from a given group B serotype will only inhibit the bactericidal reaction of an antiserum of the corresponding serotype. In addition, this extract can often be shown to have a single immunoprecipitable antigen by agar gel diffusion. Based upon these findings, we conclude that the antigens extractable by acid or saline are the same antigens to which the bactericidal activity is directed. As previously discussed, these antigens may have multiple antigenic determinants.

The specificity of the precipitin reaction depended upon the proper production of the strain extract. If an extract was allowed to remain on the cells for 1 to 2 hr after extraction or if the cells were partially autolyzed, then a characteristic nonspecific flocculation occurred in all serotype antisera when the extract was subsequently tested for precipitin activity. This flocculation was in addition to the type-specific precipitation and was seldom evident after the 2 hr of incubation at 37 °C but usually appeared only after overnight refrigeration as a large translucent flocc which seldom settled to the bottom of the capillary. This is in contrast to the type-specific precipitate, which was a fine dense precipitate evident at 2 hr and readily settled to
the bottom of the capillary after overnight refrigeration at 4°C.

A few hyperimmune antisera precipitated rather strongly (3+ to 4+) in many serotype extracts giving a typical fine dense precipitate. However, the type-specific precipitin reaction always appeared first and was a strong reaction. Recent evidence in our laboratory suggests that the extensive cross-reactivity in the case of hyperimmune antisera is due to a common antigen distinct from the serotype antigen. When these hyperimmune antisera were adsorbed for 1 hr at 37°C with one of the cross-reacting heterologous group B strains, the precipitins against the heterologous strains were removed. The homologous type-specific precipitin reaction remained.

The ability to serotype meningococci will aid in the epidemiological study of meningococcal infections since there is little definitive evidence on the spread of different meningococcal strains within populations. Our serotyping method may be used to determine whether there are certain strains which tend to cause more severe infections or to be associated with outbreaks of meningococcal disease. We now have some evidence to suggest that certain group B serotypes are more frequently associated with cases of disease and other serotypes more associated with the carrier state. This is in agreement with a recent study of group C meningococcal case strains (4).

The role of serotype antigens in the protection against group B meningococcal disease remains to be investigated. There are, however, some indications in the literature that protection against group B meningococcal disease may be at least partially type-specific. In 1967 (17) two instances were reported in which persons suffered two separate episodes of meningococcal disease. In both cases, the second episode was confirmed as due to group B. However, the first episodes may well have been caused by group B strains since group B was by far the most prevalent group at that time. Vedros et al. (18) reported a patient who had two separate attacks of group B meningococcal disease due to two antigenically distinct strains. Thus the type-specific antigens of group B meningococci may well be involved in the protection against group B meningococcal disease.

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LITERATURE CITED