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Outer Membrane Antigens of *Neisseria meningitidis* Group B Serotype 2 Studied by Crossed Immunoelectrophoresis

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This study shows that the capsular polysaccharide, protein, and lipopolysaccharide antigens from the outer membrane of *Neisseria meningitidis* group B serotype 2 may be identified by crossed immunoelectrophoresis. By using this technique, seven precipitates were resolved when outer membrane preparations were reacted against goat anti-whole cell group B type 2 antiserum. Most of these precipitates were identified by comparison with purified reference preparations. Different outer membrane preparations, reflecting different growth conditions, varied in their compositions of lipopolysaccharide, protein, and polysaccharide. Detergent treatment altered the protein and lipopolysaccharide precipitation patterns. In the presence of detergent, the lipopolysaccharide did not precipitate, and the electrophoretic migration of the protein antigens decreased. Crossed immunoelectrophoresis is a useful qualitative method for analysis of the antigenic components of the meningococcal outer membrane. The crossed immunoelectrophoresis with intermediate gel technique is presently being used to measure the human immune response to the different cell surface components.

Immunity to meningococcal disease is conferred by bactericidal antibodies. Bactericidal activity has been demonstrated against the group-specific capsular polysaccharides (11), the type-specific outer membrane proteins (4, 22), and lipopolysaccharides (LPSs) (18, 23). For meningococcal groups A and C, anticapsular antibodies, elicited by natural exposure or by immunization, are bactericidal and confer protection (12). Group B anticapsular antibodies develop in patients as a result of disease and in carriers (14, 28). In contrast, the purified group B polysaccharide does not appear to be immunogenic in humans (25). The immune response to noncapsular surface antigens has received less attention, even though antibodies to some of these antigens are bactericidal and therefore may be protective (9). These antigens have recently been studied as potential immunogens for preventing group B meningococcal disease (9, 27).

Group B meningococci have been subdivided on the basis of their outer membrane proteins into 15 to 18 serotypes (6, 26), which are shared among different serogroups (6). One of the serotypes, serotype 2, is associated with approximately 50% of meningococcal disease caused by groups B, C, Y, and W135 (5, 6, 10, 19). The purpose of this study was, therefore, to characterize the outer membrane antigens of group B serotype 2 meningococci. Crossed immunoelectrophoresis (IE) was chosen because the specificity and antibody titers in human sera toward different antigens could be studied simultaneously. Protein, LPS, and capsular polysaccharide precipitin lines were found and identified by using reference antigen preparations.

MATERIALS AND METHODS

Strains and serotype antigens. The meningococcal strains used were M986 (group B, type 2, 7) (5) and a naturally occurring noncapsular variant of M986 selected by using an antiserum agar technique, which was designated M986 NCV-1. Different serotype antigen (STA) preparations from M986 (designated a, b, c, and d) and from M986 NCV-1 (designated e, f, g, and h) were examined. The cells were grown overnight in tryptic soy broth (Difco Laboratories, Detroit, Mich.) or in modified Frantz medium B (8). Table 1 summarizes the growth conditions and buffers used to extract the STAs. The STAs were prepared from extracts by ultracentrifugation, as described previously (7). Samples of STA-a, -b, and -g were mixed with Triton X-100 (final concentration, 1%), Emulphogene BC-720 (final concentration, 2%), and/or urea (final concentrations, 1.5 to 5 M).

Reference antigen preparations. LPS was purified by detergent solubilization from strain M986 NCV-1 STA. The STA at a concentration of 1 to 2 mg/ml was treated with 2% (wt/vol; final concentration) sodium deoxycholate (Sigma Chemical Co., St. Louis, Mo.) in 0.01 M tris(hydroxymethyl)aminomethane-hydrochloride-0.01 M ethylenedinitriolotetraacetic acid buffer (pH 8.5) for 15 min at room

TABLE 1. *Growth and isolation conditions used in the preparation of STAs from group B serotype 2 meningococcal strains*

Strain	Antigen[a]	Growth medium[b]	Growth temp (°C)	Shaking (rpm)	Aeration (liters of air/min)	Extraction buffer	Final concn (mg of protein/ml)
M986	STA-a	TSB	36	125		LiCl-EDTA[c]	1.0
	STA-b	TSB	36	150		LiCl-EDTA	1.0
	STA-c	TSB	36	125		LiCl-CH_3COONa[d]	3.6
	STA-d	TSB	36	125		LiCl-CH_3COONa	1.0
M986 NCV-1	STA-e	TSB	36	125		LiCl-CH_3COONa	1.0
	STA-f	TSB	36	125		LiCl-CH_3COONa	1.0
	STA-g	TSB	37	200	40	LiCl-CH_3COONa	1.0
	STA-h	MF-B	26	200	40	LiCl-CH_3COONa	1.0

[a] STA-a, -b, -c, -d, -e, and -f were produced in baffled Fernbach flasks, and STA-g and -h were produced in a 45-liter fermentor.

[b] Abbreviations: TSB, tryptic soy broth; MF-B, modified Frantz medium B.

[c] 0.2 M LiCl–0.01 M ethylenedinitriloacetic acid (EDTA), pH 6.0.

[d] 0.2 M LiCl–0.1 M CH_3COONa, pH 5.8.

temperature. The material was then applied to a column (1.6 by 100 cm) of Sephadex G 150 (Pharmacia Fine Chemicals, Piscataway, N.J.), equilibrated in 0.01 M tris(hydroxymethyl)aminomethane–0.01 M ethylenedinitrilotetraacetic acid buffer (pH 8.5) containing 0.5% sodium deoxycholate. The LPS eluted at a K_d of approximately 0.6 and was precipitated with 80% (vol/vol; final concentration) ethanol and recovered by centrifugation at 2,000 × *g* for 10 min. The LPS pellet was washed once with 95% ethanol, dissolved in distilled water, and pelleted by centrifugation at 120,000 × *g* for 2 h at 4°C. The LPS was redissolved in water, centrifuged two additional times at 120,000 × *g*, and finally freeze-dried. This preparation contained <1% protein, as determined by amino acid analysis, and <1% nucleic acid, as determined by analysis for ribose (9).

Meningococcal group B polysaccharide (B-CHO) Lot RU-14, purified by a modified Cetavlon method (13), was obtained from Emil Gotschlich, Rockefeller University, New York, N.Y. A purified protein preparation was obtained from M986 NCV-1 by treating the STA with sodium deoxycholate (9). This protein preparation contained primarily the 41,000-dalton serotype 2 protein and approximately 5% LPS. A reference antigen from sonicated meningococci (14) was used to detect common (cross-reacting) antigens.

Crossed IE. The equipment for crossed IE was obtained from Dansk Laboratorieudstyr, Copenhagen, Denmark. The IEs were performed on glass plates (5 by 2.5 or 5 by 5 cm; Bio-Rad Laboratories, Richmond, Calif.), using 1% agarose gel (Indubiose A 37; l'Industrie Biologique Francaise, France) in barbital buffer (pH 8.6; ionic strength, 0.02), resulting in an electroendosmosis (-Mr) of 0.23. The first dimension electrophoresis was at 10 V/cm for 17 or 27 min for 2.5- and 5-cm plates, respectively, and second-dimension electrophoresis was at 1 to 2 V/cm overnight. For crossed IE, 2 μg of STA protein or reference preparation was used for the first dimension, except for B-CHO, which was used at 0.5 μg. The intermediate gel for crossed-line IE contained either no antigen or test antigen at concentrations between 0.8 and 33.3 μg/cm^2.

Antiserum was raised in a goat (no. 1064) against formalin-inactivated M986 (group B, type 2) cells. A 5-h growth in 400 ml of tryptic soybroth was inactivated with 0.5% (vol/vol) Formalin and then pelleted and suspended in 20 ml of 0.15 M NaCl. The animal received three 0.5-ml subcutaneous injections during week 1 and one during week 2. Two additional 0.5-ml doses were given intraveneously during week 2. Three 1.0- and 2.0-ml intravenous injections were given during weeks 3 and 4, respectively. The animal was bled at 3 days and again at 1 week after completion of immunization. Antiserum obtained after a second course of immunization was used in this study and incorporated in the reference gel at 26.7 μl/cm^2. Rabbit reference antibody was made by immunization with reference antigen (14).

The antigen preparations were run against an antiserum in crossed IE (24), tandem crossed IE (16), or crossed-line IE (17), with the antigen in question included in the intermediate gel between first- and second-dimension electrophoresis (absorption of antibodies in situ).

RESULTS

STA precipitation pattern. Serotype 2 STA-a and STA-b were tested against goat M986 antiserum by crossed IE (Fig. 1A and C). There were seven precipitin lines in STA-a, as enumerated in Fig. 1B. Another representative precipitation pattern is shown for STA-b (Fig. 1C). This STA preparation had all of the antigens seen in STA-a except line II, which was only irregularly present in the different purified STA. As can be seen, STA-b had less of antigens III and IV, giving better resolution of these two lines. The precipitation patterns of STA-a and STA-b were consistent from day to day when they were run against the goat antiserum. The precipitin lines were identified by comparison with various reference preparations. Line I was identified as group B polysaccharide (Fig. 2).

Addition of purified B-CHO to the STA preparation increased exclusively the area under precipitate I (data not shown). When B-CHO was added in crossed-line IE, precipitate I was elevated (Fig. 2B). A reaction of identity with B-CHO and precipitate I from STA was shown in tandem crossed IE, when B-CHO precipitated cathodally to precipitate I (Fig. 2C). When the antigens were reversed in the wells, so that B-CHO precipitated anodally to precipitate I, a reaction of partial identity was observed between these two precipitates (Fig. 2D). The B-CHO migrated more slowly than the polysaccharide in the STA and precipitated in a sharper line than the STA antigen. Both B-CHO and the B polysaccharide in the STA precipitated in a sharper cathodal line than anodal line. In addition, close examination of both B-polysaccharide precipitates revealed two closely spaced lines (Fig. 2C, arrows), indicating that at least two antigenically different populations of molecules were involved in the precipitation reaction.

The reference LPS preparation precipitated in crossed IE as two closely spaced lines (Fig. 3A). Tandem crossed IE (Fig. 3B) showed reactions of identity between purified LPS and STA precipitates III and IV. Crossed-line IE with LPS in the intermedite gel (Fig. 3C) elevated these two precipitates and revealed an additional raised horizontal line, indicating a third antigen in the LPS preparation. Precipitate VII also changed considerably since it became fainter or disappeared, and another precipitate appeared near the antigen well.

The purified reference protein preparation was only partially water soluble. When run as antigen in crossed IE, it gave rise to a faint line in approximately the same position as precipitate II, but was found to be antigenically distinct from the precipitate II antigen. In crossed-line IE with the protein reference preparation in the intermediate gel, the most pronounced elevation was observed for precipitate VI (Fig. 4B). A horizontal line was also seen, which may correspond to the soluble protein responsible for the faint precipitate in crossed IE of the reference protein preparation. The reference protein also caused a very subtle elevation of lines III, IV, V, and VII and was the only reference preparation to alter the position of precipitate V when incorporated in the intermediate gel.

Precipitate II was faint and not always present. The migration of the antigen in precipitate II was similar to that of a common meningococcal antigen (reference antigen 19) described by Hoff and Høiby (14); however, no cross-reaction could be demonstrated between precipitate II and any of the antigens in the reference antigen of Hoff and Høiby. This reference antigen preparation was a sonicate of several meningococcal strains and contained at least 65 different antigens, probably including most of the major cytoplasmic antigens. Therefore, lack of cross-reactivity between precipitate II and the reference antigen preparation suggests that antigen II is not of cytoplasmic origin.

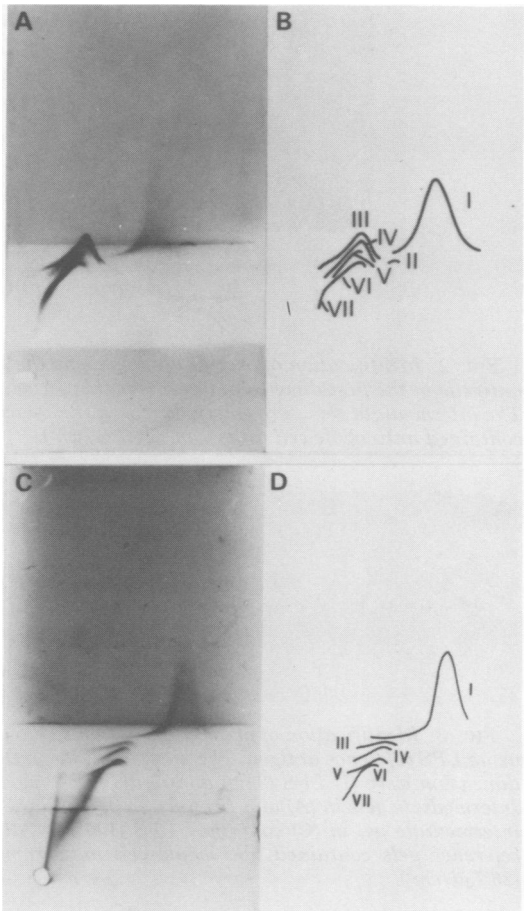

FIG. 1. *Enumeration of precipitin lines after crossed IE of two representative serotype 2 STA preparations. (A) STA-a (2 μg) reacted against goat anti-whole cell antiserum (26.7 μl/cm²). The intermediate gel is empty. Seven lines can be seen and are drawn and labeled on (B) (C and D) STA-b (2 μg) run and drawn exactly as for STA-a. For this figure and all others, the anodes were to the right (first dimension) and top (second dimension).*

Preliminary studies of STAs from group B non-type 2 strains showed that these heterologous STAs could absorb all of the goat anti-M986 antibodies against the type 2 STA-a antigen except those in precipitates V, VI, and VII. These three lines may therefore represent type 2-specific antigens.

Differences between STA preparations.

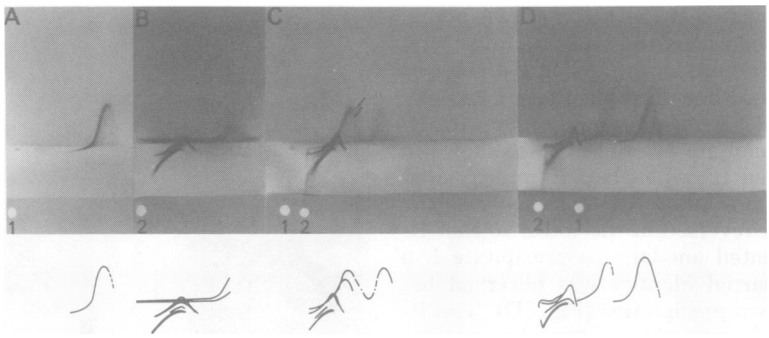

FIG. 2. *Identification of precipitate I by using the group B capsular polysaccharide reference antigen. The antigens in the first dimension were (1) meningococcal group B polysaccharide (0.5 μg) and (2) STA-a (2 μg). The intermediate gels were empty, except in (B), which contained B polysaccharide (0.2 μg). All reference gels contained anti-whole cell antiserum (26.7 μl/cm²).*

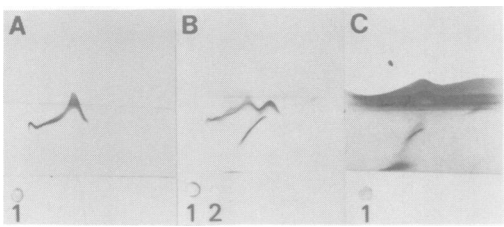

FIG. 3. *Identification of precipitates III and IV by using LPS reference antigen. The antigens in the first dimension were (1) LPS (2 μg) and (2) STA-a (2 μg). Intermediate gels in (A) and (B) were empty, and the intermediate gel in (C) contained LPS (100 μg). All reference gels contained anti-whole cell antiserum (26.7 μl/cm²).*

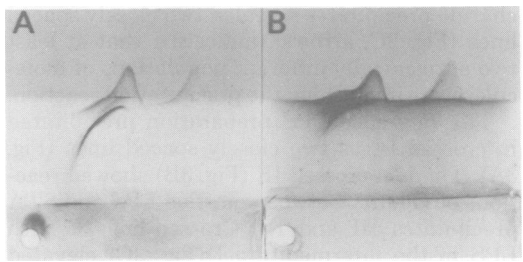

FIG. 4. *Identification of precipitate VI by using the reference protein preparation. The antigen wells contained STA-a (2 μg). The intermediate gel in (A) was empty and in B contained the reference protein preparation (40 μg). The reference gels contained anti-whole cell antiserum (26.7 μl/cm²).*

Four STAs prepared from the capsular strain and four from the noncapsular variant of M986 were studied by the various crossed IE methods (Fig. 5). The precipitation patterns of STA-b, -c, and -d prepared from the capsular strain resembled the STA-a precipitation pattern (Fig. 5A, B, and C, gels on the left of each pair). When sufficient amounts of STA-b, -c, or -d were incorporated into the intermediate gel, they were able to absorb all of the goat antibodies against STA-a (Fig. 5A, B, and C, gels on the right of each pair). Some preparations, however, contained small amounts of an additional antigen precipitating near precipitate II. This antigen was shown to be identical to the common meningococcal reference antigen, no. 19 (14). When STAs were purified by using only two ultracentrifugation steps, antigen 19 and several other additional antigens were found.

STA-e and -f were prepared from M986 NCV-1 in exactly the same manner and had identical precipitation patterns (Fig. 5D; only STA-f shown). Precipitates II through VII were discernable, whereas the group B polysaccharide precipitate was absent, as expected. Another feature of these NCV-1 STAs was the flatness of LPS precipitates III and IV. When incorporated into the intermediate gel, both STA-e and -f absorbed all antibodies against STA-a except those against the group B polysaccharide.

STA-g and -h were prepared from cells grown in a 45-liter fermentor. The cells, from which STA-g was prepared, were grown with high aeration and contained an abundance of LPS, but lacked the group B polysaccharide and at least one other antigen found in STA-a, as shown by absorption in situ (Fig. 5E). STA-h was prepared from cells grown at 26°C (slow growth rate) and contained an abundance of protein corresponding mostly to precipitate VI but very little of the other antigens. Accordingly, adsorption in situ with STA-h did not absorb all of the goat antibodies against STA-a (Fig. 5F).

Treatment of STA with nonionic detergents. Triton X-100 was used for solubilization of the LPS-protein and protein-protein complexes in STA-a. When Triton X-100 was added

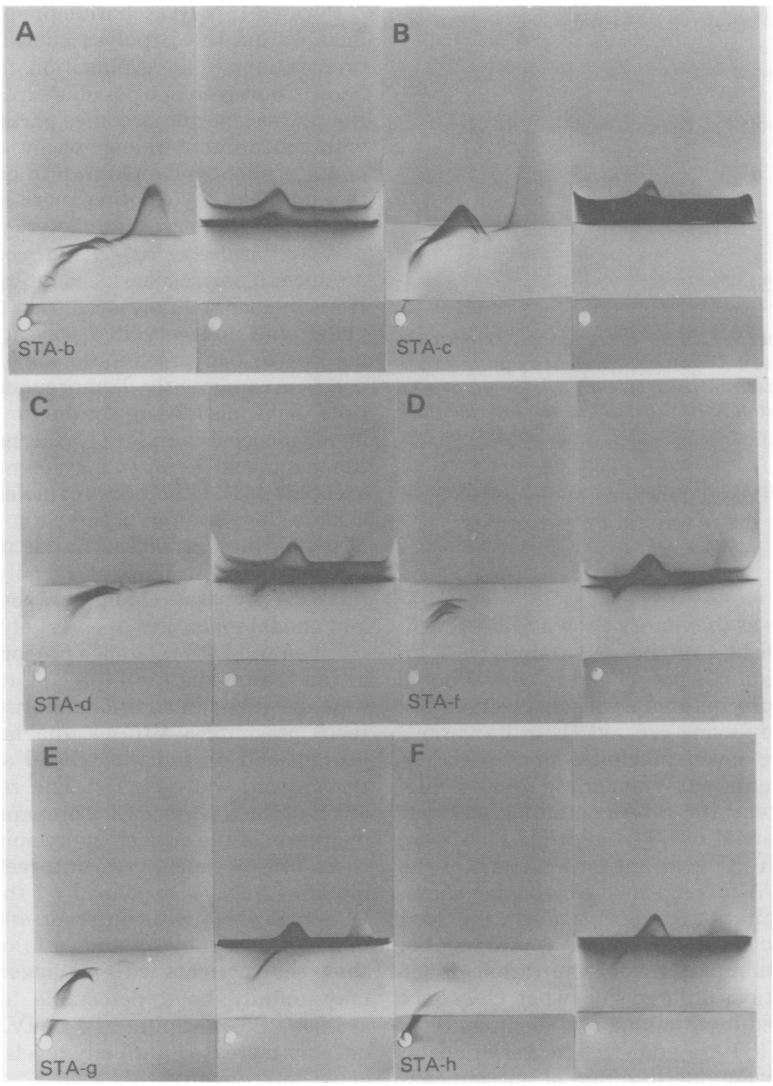

FIG. 5. *Each set of two plates (A through F) shows a different preparation of serotype 2 antigen (2 μg/plate) reacted against anti-whole cell antiserum (left plates) and the same STA incorporated into the intermediate gels (20 μg) between STA-a reacted against the antiserum (right plates).*

to STA-a (Fig. 6A), but not to the gel, precipitates III and IV, corresponding to LPS, became flat and easily distinguishable as two precipitates. Precipitates VI and VII also became more separated. Addition of Triton X-100 to the gel (Fig. 6B) reduced the number of precipitates from seven to four or five. Precipitates I and II were easily identified, as they were essentially unaffected by the detergent, whereas precipitates III and IV disappeared when detergent was present in the gel. Likewise, the reference LPS preparation did not produce precipitates when run in detergent-containing gel, probably due to detergent-induced depolymerization of the LPS. The remaining three precipitates (V, VI, and VII) appeared as two or three lines near the antigen well (Fig. 6B). These latter precipitates could be better resolved in the detergent gels by increasing the antigen concentration 10- to 15-fold, which resulted in four to six bell-shaped precipitates (data not shown). Essentially the same changes were seen for the other STAs examined and when Emulphogene BC-720 was substituted for Triton X-100. The presence of

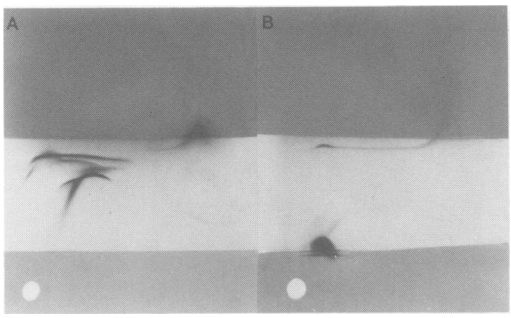

FIG. 6. *Crossed IE in the presence of detergent.(A) STA-a (2 μg) in 1% Triton X-100 reacted against anti-whole cell antiserum (26.7 μl/cm²). (B) Same as (A), but with 1% Triton X-100 also in the gel. Intermediate gels were empty.*

urea in the STA preparations or the gel or both did not affect the precipitation patterns.

DISCUSSION

The STA was previously shown to be a high-molecular-weight protein-LPS complex (7). In double diffusion in agar gel and in conventional IE, the STA precipitated as one or two lines (7). In contrast, by using crossed IE in its various modifications, seven precipitin lines were detected and identified. When crude lithium chloride-sodium acetate STA-containing extracts were used instead of STA, multiple lines were observed with the goat antiserum. Each of the individual STA preparations tested gave a consistent precipitation pattern against the goat antiserum. However, the general appearances of the precipitation patterns for the various M986 STA preparations differed somewhat. Only once did we succeed in obtaining two identical STA preparations (STA-e and -f). The variations in the patterns were due to different antigen concentrations and may have been due to differences in the state of the antigens. These differences may be related to culture conditions, which are known to influence the composition of the outer membrane (8), or to the STA extraction conditions.

The seven lines in the STA-a preparation were identified as follows: line I, group B polysaccharide; line II, minor cell envelope protein; lines III and IV, LPSs; and lines V, VI, and VII, major outer membrane proteins. The B polysaccharide was not previously demonstrated in the STAs prepared as described (7), which were believed to be free of capsular polysaccharide contamination earlier. Another unexpected observation was that most of the LPS gave precipitin lines separate from the proteins.

Purified B polysaccharide precipitated in two lines, as did the B polysaccharide in the STA preparations. The explanation for this is not known; however, one possible explanation is that the polysaccharide becomes partially degraded, with concomitant change of antigenic determinants, a phenomenon known to occur with protein antigens (2). Another more likely explanation is that there are microheterogeneities in the polysaccharides. *Escherichia coli* K1, which produces a polysaccharide identical to the group B meningococcal polysaccharide (15), has acetylated and nonacetylated polysaccharides, giving rise to two precipitation lines (20). Differences in apparently homogenous polysaccharides have also been shown for the group C meningococcal capsular polysaccharide in which the sialic acid residues may or may not be *O*-acetylated (1). Differences in molecular size may explain the variation observed in the sharpness of the purified B-polysaccharide precipitin lines as compared with those of the polysaccharide in the STA preparation and between the cathodal and anodal molecules of each polysaccharide.

When the LPS reference preparation was run in the intermediate gel, precipitates III and IV were elevated, an additional horizontal line was seen, precipitate VII became faint or disappeared, and an indistinct band appeared near the antigen well (Fig. 3). The reason for this effect of the reference LPS preparation upon the migration of the antigen in precipitate VII is not clear. The presence of two or three LPS-containing lines may be explained by the presence of LPS molecules with different antigenic determinants. Preliminary studies in our laboratory show that patients with meningococcal disease may initally have detectable antibody only against LPS precipitate III or IV, but go on to develop increased antibody levels to both antigenic determinants.

The STA protein reference preparation, containing primarily the 41,000-dalton serotype 2 determinant protein, markedly elevated precipitate VI and gave rise to a spur over precipitate VII. Absorption in situ experiments with this reference protein preparation in the intermediate gel indicated that even if there are shared antigenic determinants in precipitates V, VI, and VII, the 41,000-dalton protein was present mostly in precipitate VI.

Electron microscopy shows that the STA consists of membrane vesicles identical to the cell wall blebs reported by DeVoe and Gilchrist (3). These particles contain phospholipid as well as protein and LPS. We were, therefore, surprised to find that the LPS was largely separate from the protein, as shown by the immunoprecipita-

tion patterns. It is possible that the electrophoresis per se disrupts the vesicles, resulting in largely separate protein and LPS molecules.

Detergent treatment of the STA resulted in four to five rather than seven precipitin lines. This lesser number was due mainly to the disappearance of the two LPS-containing precipitates (III and IV). Detergent causes depolymerization of the LPS from $>10^6$ daltons to approximately 10^4 daltons (18). The low-molecular-weight LPS subunits were apparently no longer able to precipitate in the gel. Precipitates I and II were essentially unaffected by the detergent, which suggests that the responsible antigens do not contain large hydrophobic regions and are therefore probably not part of the outer membrane lipid bilayer as such. The precipitate I antigen which is the capsular polysaccharide is exterior to the outer membrane. The structural location of precipitate II antigen is unknown but may be a loosely bound or periplasmic space protein. The resolution of precipitates V, VI, and VII at low antigen concentrations did not improve in the presence of detergent. Better resolution may, however, be obtained by using an agarose having less electroendosmotic flow (21). Smyth and Salton also increased the resolution of gonococcal outer membrane antigens by increasing the antigen concentration considerably. Likewise, when we increased the concentration of meningococcal STA from 2 to 25 μg in the presence of detergent, we obtained good resolution with bell-shaped precipitin lines.

Crossed-line IE is now being used to analyze the content of outer membrane antigens in various other antigen preparations. In addition, crossed IE with intermediate gel is being used to study antibodies in human sera to multiple meningococcal surface antigens by using the goat whole cell antiserum as reference. We are studying the antibody response against LPS, protein, and capsular polysaccharide in patients, carriers, and vaccinees. The assay system as described above in absence of detergent, where the LPS lines are present and separated from the protein and capsular polysaccharide lines, has proven most suitable for these antibody determinations.

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LITERATURE CITED

1. **Apicella, M. A.** 1974. Identification of subgroup antigen on the *Neisseria meningitidis* group C capsular polysaccharide antigen. J. Infect. Dis. **129**:147-153.
2. **Bjerrum, O. J., J. Ramlau, I. Clemmesen, A. Ingild, and T. C. Bøg-Hansen.** 1975. An artifact in quantitative immunoelectrophoresis of spectrin caused by proteolytic activity in antibody preparations. Scand. J. Immunol. 4(Suppl. 2):81-88.
3. **DeVoe, I. W., and J. E. Gilchrist.** 1973. Release of entotoxin in the form of cell wall blebs during *in vitro* growth of *Neisseria meningitidis*. J. Exp. Med. **138**: 1156-1167.
4. **Frasch, C. E.** 1977. Role of protein serotype antigens in protection against disease due to *Neisseria meningitidis*. J. Infect. Dis. **136**:S84-S90.
5. **Frasch, C. E., and S. S. Chapman.** 1973. Classification of *Neisseria meningitidis* group B into distinct serotypes. III. Application of a new bactericidal inhibition technique to distribution of serotypes among cases and carriers. J. Infect. Dis. **127**:149-154.
6. **Frasch, C. E., and G. L. Friedman.** 1977. Identification d'un serotype meningococcique associe a la maladie et commun aux meningocoques des groupes B, C. Y et 135W. Med. Trop. (Marseille) **37**:155-159.
7. **Frasch, C. E., and E. C. Gotschlich.** 1974. An outer membrane protein of *Neisseria meningitidis* group B responsible for serotype specificity. J. Exp. Med. **140**: 87-104.
8. **Frasch, C. E., R. M. McNelis, and E. C. Gotschlich.** 1976. Strain-specific variation in the protein and lipopolysaccharide composition of the group B meningococcal outer membrane. J. Bacteriol. **127**:973-981.
9. **Frasch, C. E., and J. D. Robbins.** 1978. Protection against group B meningococcal disease. III. Immunogenicity of serotype 2 vaccines and specificity of protection in a guniea pig model. J. Exp. Med. **147**:629-664.
10. **Gold, R., J. L. Winklehake, R. S. Mars, and M. S. Artenstein.** 1971. Identification of an epidemic strain of group C *Neisseria meningitidis* by bactericidal serotyping. J. Infect. Dis. **124**:593-597.
11. **Gold, R., M. L. Lepow, I. Goldschneider, and E. C. Gotschlich.** 1971. Immune response of human infants to polysaccharide vaccines of groups A and C *Neisseria meningitidis*. J. Infect. Dis. **136**:S31-S35.
12. **Goldschneider, I., E. C. Gotschlich, and M. S. Artenstein.** 1969. Human immunity to the meningococcus. II. Development of natural immunity. J. Exp. Med. **129**: 1327-1348.
13. **Gotschlich, E. C.** 1975. Development of polysaccharide vaccines for the prevention of meningococcal diseases. Monogr. Allergy **9**:245-258.
14. **Hoff, G. E., and N. Høiby.** 1978. Crossed immunoelectrophoretic analysis of *Neisseria meningitidis* antigens and of corresponding antibodies in patients with meningococcal disease. Acta Pathol. Microbiol. Scand. Sect. C **86**:1-9.
15. **Kasper, D. L., J. L. Winkelhake, W. D. Zollinger, B. L. Brandt, and M. S. Artenstein.** 1973. Immunological similarity between polysaccharide antigens of *Escherichia coli* 07:K1(L):NM and group B *Neisseria meningitidis*. J. Immunol. **110**:262-268.
16. **Krøll, J.** 1973. Tandem-crossed immunoelectrophoresis. Scand. J. Immunol. **2**:(Suppl. 1):57-59.
17. **Krøll, J.** 1973. Crossed-line immunelectrophoresis. Scand. J. Immunol **2**(Suppl. 2):79-81.
18. **Mandrell, R. E., and W. D. Zollinger.** 1977. Lipopolysaccharide serotyping of *Neisseria meningitidis* by hemagglutination inhibition. Infect. Immun. **16**:471-475.
19. **Munford, R. S., C. M. Patton, and G. W. Gorman.** 1975. Epidemiologic studies of serotype antigens common to group B and C *Neisseria meningitidis*. J. Infect. Dis. **131**:286-290.
20. **Ørskov, F., I. Ørskov, A. Sutton, R. Schneerson, W. Lin, W. Egan, G. E. Hoff, and J. B. Robbins.** 1979. Form variation in *Escherichia coli* K1: determined by O-acetylation of the capsular polysaccharide. J. Exp. Med. **149**:669-685.
21. **Smyth, C. J., and M. R. J. Salton.** 1977. Crossed im-

munoelectrophoresis: a new approach to high resolution analysis of gonococcal antigens and antibodies, p. 313. *In* R. B. Roberts (ed.), The gonococcus. John Wiley & Sons, Inc., New York.

22. **Tettmar, R. E., J. D. Abbott, and D. M. Jones.** 1977. Meningococcal meningitis in Royal Air Force recruits. Public Health **91**:252–257.
23. **Tramont, E. C., J. C. Sadoff, and M. S. Artenstein.** 1974. Cross-reactivity of *Neisseria gonorrhoeae* and *Neisseria meningitidis* and the nature of antigens involved in the bactericidal reaction. J. Infect. Dis. **130**: 240–247.
24. **Weeke, B.** 1973. Crossed immunoelectrophoresis. Scand. J. Immunol. **2**(Suppl. 1):47–50.
25. **Wyle, F. A., M. S. Artenstein, B. L. Brandt, E. C. Tramont, D. L. Kasper, P. L. Alteri, S. L. Berman, and J. P. Lowenthal.** 1972. Immunogenic response of man to group B meningococcal polysaccharide vaccines. J. Infect. Dis. **126**:514–522.
26. **Zollinger, W. D., and R. E. Mandrell.** 1977. Outer-membrane protein and lipopolysaccharide serotyping of *Neisseria meningitidis* by inhibition of a solid-phase radioimmunoassay. Infect. Immun. **18**:424–433.
27. **Zollinger, W. D., R. E. Mandrell, P. Altieri, S. Berman, J. Lowenthal, and M. S. Artenstein.** 1978. Safety and immunogenicity of a *Neisseria meningitidis* type 2 protein vaccine in animals and humans. J. Infect. Dis. **137**:728–739.
28. **Zollinger, W. D., C. L. Pennington, and M. S. Artenstein.** 1974. Human antibody response to three meningococcal outer membrane antigens: comparison by specific hemagglutination assays. Infect. Immun. **10**:975–984.