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Protein Fraction with Immunogenic Potential and Low Toxicity Isolated from the Cell Wall of *Neisseria meningitidis* Group B

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Several fractions were extracted from the cell envelope (CE) of *Neisseria meningitidis* group B and characterized with regard to their morphology, antigenicity, protein composition, and toxicity. Whole bacterial cells were suspended in a medium of low ionic strength and disrupted in a French pressure cell. The crude CE thus obtained were separated into cell membrane (CM) enriched and cell wall (CW) enriched fractions on sucrose density gradients. In addition, CM and CW fractions were separated from CE on the basis of differential solubility in the nonionic detergent, Triton X-100. The Triton-insoluble fraction, containing primarily CW components, was further treated with a mixture of Triton and ethylenediaminetetraacetic acid, which was shown to remove additional protein and most of the lipopolysaccharide. Electron microscope examination of the various fractions revealed typical unit membrane structures in the case of CM, or large, open segments in the case of CW. The Triton-insoluble and especially the Triton-ethylenediaminetetraacetic acid-insoluble fractions consisted of small vesicular structures. All fractions, except the Triton-soluble fraction, when assayed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, were shown to contain one major protein component accounting for more than 50% of the total. Sera from rabbits immunized with the various fractions formed precipitin lines in immunodiffusion tests against the homologous and some of the heterologous fractions. High-titer bactericidal antibodies were also demonstrated in these sera when tested against the homologous strains. Toxicity studies in rats sensitized with lead acetate indicate that the level of contamination of Triton-insoluble/Triton-ethylenediaminetetraacetic acid-insoluble fractions with lipopolysaccharide was significantly smaller than that of the other fractions.

In a previous study (10), some chemical, immunological, and enzymatic properties of the spheroplast membranes of group B *Neisseria meningitidis* were described. The spheroplast membranes were found to elicit the production of antibodies in rabbits that, in immunodiffusion tests, reacted with homologous antigen and with a cell wall (CW) antigen. It was not further tested as a possible immunogen because it was moderately pyrogenic for rabbits. In an attempt to further study the nature of the meningococcal cell envelope (CE) and to obtain CE fractions with reduced toxicity, some procedures originally designed for the extraction of CW and cytoplasmic membrane (CM) fractions from *Escherichia coli* were adapted to *N. meningitidis*.

CM- and CW-enriched fractions have been obtained from *E. coli* by Schnaitman (20) by the disruption, with a French pressure cell, of whole bacterial cells in a medium of low ionic strength and the subsequent separation of fragments of similar density on sucrose density gradients. In addition, another method was developed by Schnaitman for the separation of CM proteins from CW proteins based on their differential solubility in the nonionic detergent, Triton X-100 (22). CM proteins of *E. coli* were solubilized by Triton X-100, whereas CM proteins were not. It was also observed that Triton, which by itself dissolved 42% of the lipopolysaccharide (LPS), in the presence of ethylenediaminetetraacetic acid (disodium salt, EDTA) solubilized 87% of the remaining LPS, but only 35 to 50% of the CW protein. Further extractions with Triton amd EDTA removed no additional protein (23).

These observations on *E. coli* prompted us to study the group B meningococcus by similar procedures. CE fractions, obtained from meningococcal strain Nor-7, were extracted and compared with regard to their structure, antigenic composition, and toxicity.

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MATERIALS AND METHODS

Cultures and growth conditions. The strain of *N. meningitidis* group B (Nor-7) used in this study has been described previously (10). It was shown by C. E. Frasch (personal communication) to belong to serotype 2 (5). Frozen stock cultures were inoculated onto Mueller-Hinton agar (Difco) plates and incubated overnight (16 h) at 37 C in a humid atmosphere of 5% CO_2 in air. The growth from four plates was suspended in tryptic soy broth (Difco), and eight 1-liter side-arm flasks, each containing 250 ml of prewarmed tryptic soy broth, were inoculated with a sufficient number of cells to give an optical density reading of approximately 0.05 at 540 nm (Bausch & Lomb Spectronic 20). The flasks were incubated, while being shaken at 37 C, until an optical density of 0.8 to 1.0 was reached (usually 4 h).

Preparation of CE fractions. Log-phase cells were harvested by centrifugation at 16,000 × *g* for 10 min. The pellets were suspended and pooled in approximately 100 ml of 0.05 M tris(hydroxymethyl)aminomethane buffer (pH 7.5) containing 1 mM EDTA. This suspension was placed in a chilled 500-ml Sorvall Omnimixer blender and treated for 1 min at a speed setting of 7.5. Cells were collected by centrifugation at 16,000 × *g* for 10 min, and the supernatant was decanted. The pellet was resuspended in 15 ml of tris-(hydroxymethyl)aminomethane-EDTA buffer, and 1 mg of deoxyribonuclease and 1 mg of ribonuclease (Calbiochem) were added. The mixture was gently agitated for 1 to 2 min, and the cells were broken by two passages through a chilled French pressure cell (American Instrument Co., Silver Spring, Md.) at 20,000 psi. $MgCl_2$ was added to a final concentration of 2 mM, and the suspension was centrifuged for 5 min at 3,000 × *g* to remove intact cells and cell debris. The supernatant was removed and centrifuged for 1 h at 55,000 rpm in a Beckman 40 Ti rotor. The pellet obtained from this centrifugation constituted the crude CE fraction. To separate the CM- and CW-enriched fractions, the crude CE were suspended in 4.5 ml of 0.01 M *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 7.4) with the aid of a 7-ml Dounce tissue homogenizer. A 1.5-ml volume was layered either on 30-ml continuous sucrose gradients (70 to 40%) or on discontinuous gradients made of 70, 56, and 20% sucrose (8, 12, and 10 ml, respectively) prepared in HEPES. Gradients were centrifuged for 16 h in a Beckman SW25.1 rotor at 23,500 rpm. Continuous gradients were fractionated from the bottom of the tube with a Buchler piercing unit (Buchler Instruments, Fort Lee, N.J.). Fractions of 0.6 ml were collected and diluted to 2.0 ml with HEPES buffer for determination of absorption and enzymatic activity. When discontinuous gradients were used, visible bands were extracted with a Beckman tube slicer. The extracted bands were diluted with HEPES to reduce the sucrose concentration and centrifuged at 55,000 rpm for 1 h in a Beckman 60 Ti rotor. Samples were washed twice with HEPES, resuspended in a small volume of the same buffer, and stored at −80 C.

When CE fractionation was not required, the crude preparation was applied to a discontinuous gradient containing 20 ml of 70% sucrose and 10 ml of 20% sucrose. The tubes were centrifuged, and the complete CE band located on top of the 70% sucrose was harvested and washed as described above. The CE fraction was then extracted with Triton X-100 and EDTA by the procedures of Schnaitman (18, 22, 23). The envelopes were incubated at a protein concentration of approximately 2 mg/ml in 2% Triton X-100 and 5 mM $MgCl_2$ in 0.01 M HEPES buffer (pH 7.4) for 20 min at 23 C (room temperature) with occasional swirling. The suspensions were then chilled in an ice-water bath and centrifuged for 1 h at 50,000 rpm in a Beckman 60 Ti rotor at 4 C. The supernatant fluid, containing the Triton-soluble (TS) material was removed, and 2 volumes of cold (−20 C) 95% ethanol was added slowly while the solution was being held in a salt-ice-water bath (−15 C). The mixture was placed in a freezer at −20 C and allowed to stand overnight. The precipitated protein was recovered by centrifugation at 20,000 rpm for 30 min in a Beckman 30 rotor at 4 C. The pellets were pooled, washed three times with saline (the same centrifugation conditions), resuspended in saline, and stored in 1-ml samples at −80 C. This material, the TS fraction, presumably consisted primarily of CM protein and some CW LPS. The pellet obtained in the first centrifugation step (after Triton extraction) was designated the Triton-insoluble (TI) fraction and was either resuspended in HEPES buffer and stored at −80 C or was washed three times with saline and resuspended in saline for storage.

TI material was extracted further by suspension of the pellet to the original volume in 2% Triton X-100 and 5 mM EDTA in HEPES buffer and incubated at 23 C for 20 min. It was then chilled in an ice bath and centrifuged for 1 h at 50,000 rpm at 4 C in a Beckman 60 Ti rotor. The supernatant was aspirated, and pellets were pooled and reextracted twice with Triton X-100 and EDTA. After the third Triton-EDTA extraction, pellets were pooled and washed three times with saline by resuspension and centrifugation for 1 h at 55,000 rpm. The washed fraction, designated Triton-insoluble/Triton-EDTA-insoluble fraction (T/TEI), was resuspended in saline and stored in 1-ml samples at −80 C. Figure 1 describes schematically the fractions extracted from crude CE.

Electron microscopy. Samples of CE fractions were sedimented by centrifugation at 40,000 rpm in a Beckman type 40 rotor, and the pellets were fixed for 2 h in 2% gluteraldehyde in 0.05 M cacodylate buffer (pH 7.3). After an overnight wash with 10% sucrose in 0.05 M cacodylate, the pellets were divided into 1-mm cubes and postfixed in the cacodylate buffer containing 1% OsO_4 and 10% sucrose. The cubes were dehydrated through graded concentrations of ethanol, block-stained in 1% uranyl acetate in 100% ethanol, and embedded in Epon. Sections were cut with a Porter-Blum MT-2 microtome (Sorvall Co.), double-stained with uranyl acetate and lead citrate (17), and viewed in a Siemens IA electron microscope operated at 80 kV.

SDS-polyacrylamide gel electrophoresis. Solubilization of the CE fractions and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis were by the methods of Sabet and Schnaitman (18). CE fractions were dissolved by suspending them at a

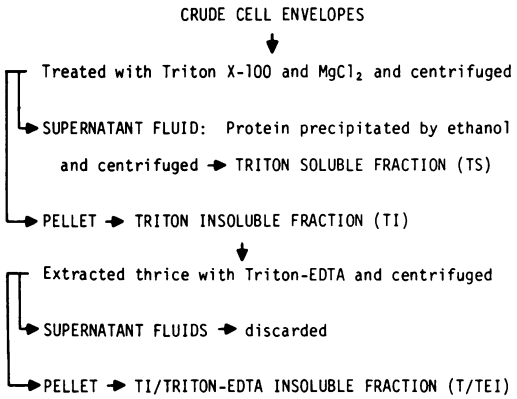

FIG. 1. *Schematic representation of the fraction extracted from crude cell envelopes.*

protein concentration of 3 to 5 mg/ml in a solution of 8 M urea, 1% SDS, 0.1% 2-mercaptoethanol, and 0.5 mM EDTA in 0.1 M sodium phosphate buffer (pH 7.2) and incubating this mixture in screw-capped tubes under nitrogen at 37 C for 2 h. The samples were then dialyzed overnight under nitrogen in a solution containing 8 M urea, 0.5 mM EDTA, 0.1% SDS, and 0.1% 2-mercaptoethanol made up in 0.1 M sodium phosphate buffer (pH 7.2). The dialyzed fractions were placed in a boiling-water bath for 3 to 5 min and centrifuged at 40,000 rpm in a Beckman type 40 rotor for 1 h to remove unsolubilized material. The solubilized samples were stored frozen at −20 C.

Samples were applied, in 10- to 50-μliter amounts, to 7.5% SDS-polyacrylamide gels (5 mm in diameter and approximately 9 cm long) prepared by the procedure of Maizel (16). A 10-μliter volume of 0.05% bromophenol blue (marker dye) was added to approximately 0.3 ml of sample prior to application. The gels were run at 5 mA/gel for 8 h and were stained overnight and destained in a Hoeffer diffusion destainer with solutions described by Swank and Munkres (24). Stained gels were scanned in a Beckman Acta III spectrophotometer with a gel scanning attachment.

Molecular weight determinations were also performed in the SDS-polyacrylamide gels described above. Standard proteins (lactic dehydrogenase, catalase, bovine serum albumin, cytochrome *c*, ribonuclease, and aldolase) were dissolved in the dialysis mixture, and the unknown molecular weight was calculated according to Weber and Osborn (25).

Chemical and immunological methods. LPS was isolated from the Nor-7 strain of meningococci by the procedure of Westphal and Jann (26). Protein was determined by the method of Lowry et al. (15), with Dade Lab-trol (American Hospital Supply Corp., Miami, Fla.) as a standard. Enzyme assays and immunodiffusion tests in agarose gels were performed as described previously (10), using rabbit antisera against the CE fractions prepared by the procedure of Fukui et al. (6).

Bactericidal test. Frozen stock cultures of meningococci were inoculated onto Mueller-Hinton agar plates and incubated overnight at 37 C in 7% CO_2. A loopful of growth was transferred to a 125-ml Erlenmeyer flask containing 25 ml of Mueller-Hinton broth; the flask was then incubated for 4 h in a shaking water bath (200 rpm) at 37 C. The cell suspension containing approximately 10^9 colony-forming units per ml was diluted to 10^4 colony-forming units per ml in Dulbecco balanced salt solution (Grand Island Biological Co.) containing 0.1% gelatin. This constituted the cell suspension for the bactericidal test.

Normal rabbit serum was used as a source of complement for all bactericidal assays. Sera to be assayed for bactericidal antibody were heat-inactivated for 30 min at 56 C, diluted in Dulbecco balanced salt solution containing 0.1% gelatin, and held in an ice-water bath until used. The bactericidal test mixture consisted of 0.1 ml of meningococcal cell suspension, 0.1 ml of normal rabbit serum, 0.1 ml of the desired test antiserum dilution, and 0.7 ml of Dulbecco balanced salt solution containing 0.1% gelatin. Control tubes were used containing only normal rabbit serum, buffer, and cells. After incubation of the tubes at 37 C in a shaking water bath for 45 min, triplicate 0.1-ml samples were removed and plated on Mueller-Hinton agar. Colonies were counted after overnight incubation in a 7% carbon dioxide atmosphere at 37 C. Bactericidal titers were expressed as the reciprocal of the serum dilution killing 90% of the meningococci in comparison with the normal rabbit serum control.

Toxicity testing. Toxicity was tested by demonstration of the Shwartzman reaction in rabbits and lethality of rats sensitized with lead acetate. Shwartzman tests were performed by injecting various levels of the preparation, in 0.1-ml volumes, intradermally on the shaved backs of New Zealand rabbits, followed 24 h later by injection of 50 μg, in a 0.5-ml volume, of the same material intravenously. The severity of the skin reactions were observed for 3 days. Rats weighing 180 to 220 g were injected intravenously with 10 μg of lead acetate, and this injection was immediately followed by intravenous injection of the fraction to be tested, as described by Jones et al. (11). Ten rats were used for each dilution, and the statistical implications of the results were analyzed by the procedure of Litchfield and Wilcoxon (14).

RESULTS AND DISCUSSION

Separation of CE fractions. The disruption of *N. meningitidis* cells with a French pressure cell in a medium of low ionic strength yielded small CE fragments that could be separated by sucrose density centrifugation. Figure 2 illustrates the distribution of material absorbing at 280 nm and the corresponding succinic dehydrogenase activity after centrifugation of the crude CE fraction from strain Nor-7 on a continuous 40 to 70% sucrose gradient. Two visible bands of turbidity were observed corresponding to the two absorbance peaks shown in Fig. 2. The upper band had a very strong reddish-brown cast, whereas the lower band was white. Although a low level of activity was seen in the lower peak, most of the succinic dehydrogenase

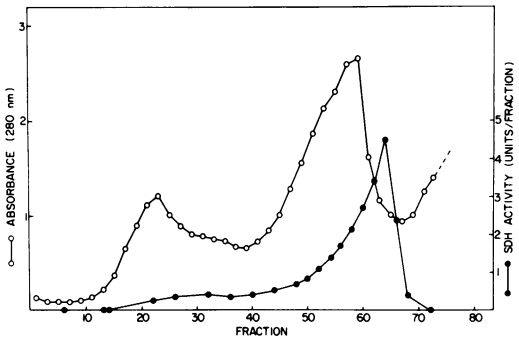

FIG. 2. *Fractionation of crude envelope fraction on a 40 to 70% (wt/vol) continuous sucrose gradient. The sucrose was dissolved in 0.01 M HEPES buffer (pH 7.4). Fractions of 0.6 ml were collected from the bottom of the tube.*

activity (a marker for the cytoplasmic membrane) was found in the area of the reddish visible band. The succinic dehydrogenase peak appeared slightly above the upper 280-nm absorbance peak, suggesting that enzymatic activity is more highly concentrated in the smaller fragments of the band detected by absorbance. When discontinuous gradients (70, 56, and 20% sucrose) were used for the separation of larger amounts of material, whole cells sedimented to the bottom of the tube, the CW-enriched fraction was layered at the 70% interface, and the CM-enriched fraction was layered at the 56% interface. The results obtained were entirely comparable to those described by Schnaitman (22) for *E. coli*. When crude CE fractions were extracted with Triton X-100, the soluble material, precipitated by ethanol, was reddish-brown in color, indicating that, as in the case of *E. coli* (22), this procedure concentrated CM protein. The TI fraction and the T/TEI fraction derived from TI were white, as was the crude CE-enriched fraction. The yield from 1 liter of culture or 1 g (wet weight) of whole cells was approximately 17 mg of CE, but the total yield was somewhat less when CM and CW were separated, 4 and 5 mg, respectively. The yield of T/TEI obtained directly from CE was about 4 mg.

Electron microscopy. The morphology of the CM-enriched and CW-enriched fractions are shown in Fig. 3A and B, respectively. The CM fraction contained primarily small, closed vesicles consisting of single unit membranes. The CW fraction, on the other hand, consisted of large, open fragments with either a single unit membrane or the typical five-layered structure of the CE. Electron micrographs (not shown) of the crude CE preparation from which these fractions were obtained showed mixtures of the two types of structure.

The TS fraction is shown in Fig. 4A and appears to be a mixture of intact CM vesicles, sections of unit membrane (probably of CM origin), and protein aggregates. These aggregates were possibly micelles mixed with detergent, as suggested by Schnaitman (22).

Fig. 4B and C illustrate the morphology of the TI and T/TEI fractions, respectively. The micrographs show small vesiculated structures consisting of single unit membrane layers after Triton X-100 treatment and similar, but less defined, structures after Triton-EDTA treatment. Although it is most likely that these structures were of CW origin, as indicated by their antigenic properties discussed below, they were quite different from the large, irregularly shaped membranes observed in the case of the CW-enriched fraction (Fig. 3B) or the corresponding structures of *E. coli* (22). It appears, therefore, that treatment with Triton X-100, and in particular with Triton-EDTA, weakened the CW to the extent that small vesicles were formed more closely resembling CM than CW.

SDS-polyacrylamide gel electrophoresis. Optical density scans of the stained SDS-polyacrylamide gels of the meningococcal CE fractions are shown in Fig. 5. The complete CE fraction contained one major protein component and at least two other components of similar electrophoretic mobility. The major component was also seen in the CM, CW, the TI, and the T/TEI fractions, whereas the levels of the other components were greatly reduced. The TS fraction showed several bands, none of which constituted a major portion of the total protein. The presence of a single major peak in gels of the meningococcal envelope, wall, and TI fractions is similar to the observations made by Schnaitman for *E. coli* (19, 20, 22, 23). In a survey of the electrophoretic patterns of CE of various gram-negative bacteria, Schnaitman (21) found that *Neisseria catarrhalis* and *Pseudomonas aeruginosa*, unlike *E. coli* and the other gram-negative organisms studied, showed two major protein components. It is not surprising to find a difference between *N. catarrhalis* and *N. meningitidis* in view of the evidence indicating that *N. catarrhalis* is not closely related to the other members of the genus *Neisseria* (2, 9, 12, 13), leading to the proposal that it be transferred to a new genus, *Branhamella* (2).

The molecular weight of the major protein from the meningococcal CE was estimated by gel electrophoresis to be 40,000. This corresponds to the molecular weight of 44,000 reported by Schnaitman (23) for the major protein component of the *E. coli* CW.

Antigenic properties. Figures 6 and 7 show

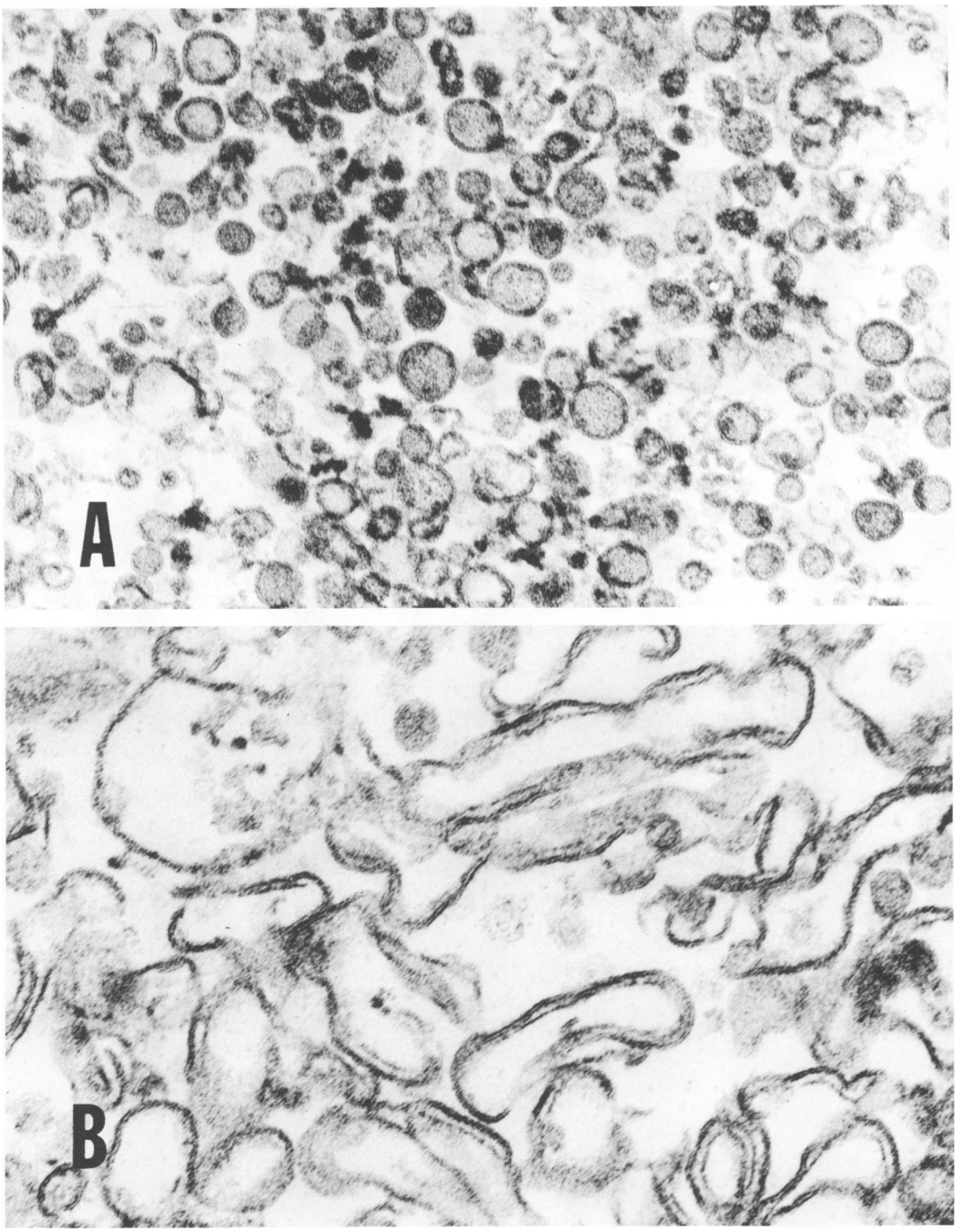

FIG. 3. *Electron micrographs of fractions obtained by density gradient centrifugation of crude cell envelopes. (A) Cytoplasmic membrane-enriched fraction (CM); magnification, ×90,000. (B) Cell wall-enriched fraction (CW); magnification, ×114,000.*

the immunodiffusion patterns obtained when rabbit antisera against CM, CW, TS, and T/TEI fractions were made to react against these fractions and unfractionated CE.

The cross-contamination of antigens in the CW and CM fractions is evident in Fig. 6A and B, in which CM and CW antisera reacted against these fractions and against CE. At least

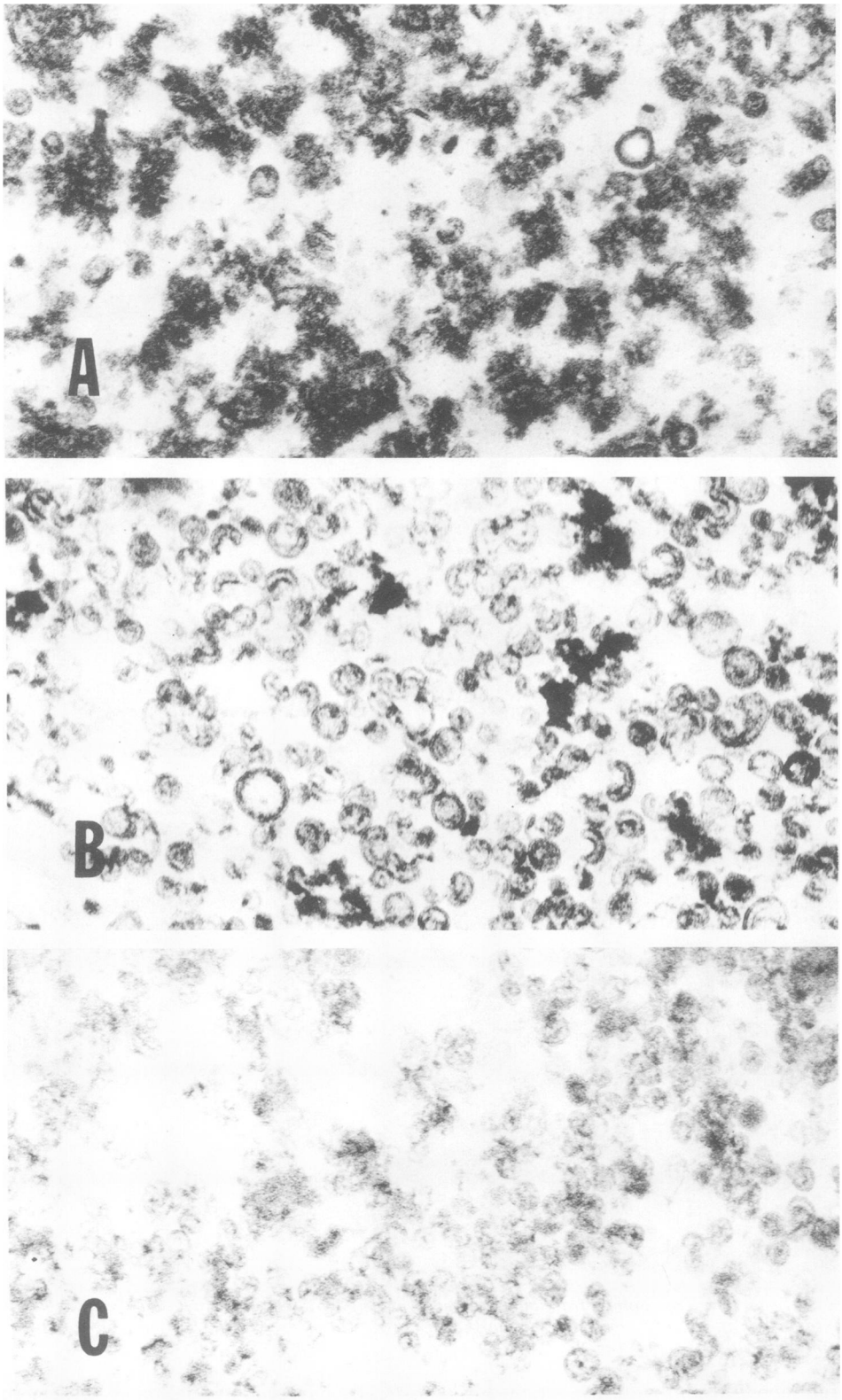

FIG. 4. *Electron micrographs of fractions obtained by treatment of crude cell envelopes with Triton X-100 and EDTA. (A) Triton-soluble fraction (TS); (B) Triton-insoluble fraction (TI); (C) Triton-insoluble/Triton-EDTA-insoluble fraction (T/TEI). All magnifications ×90,000.*

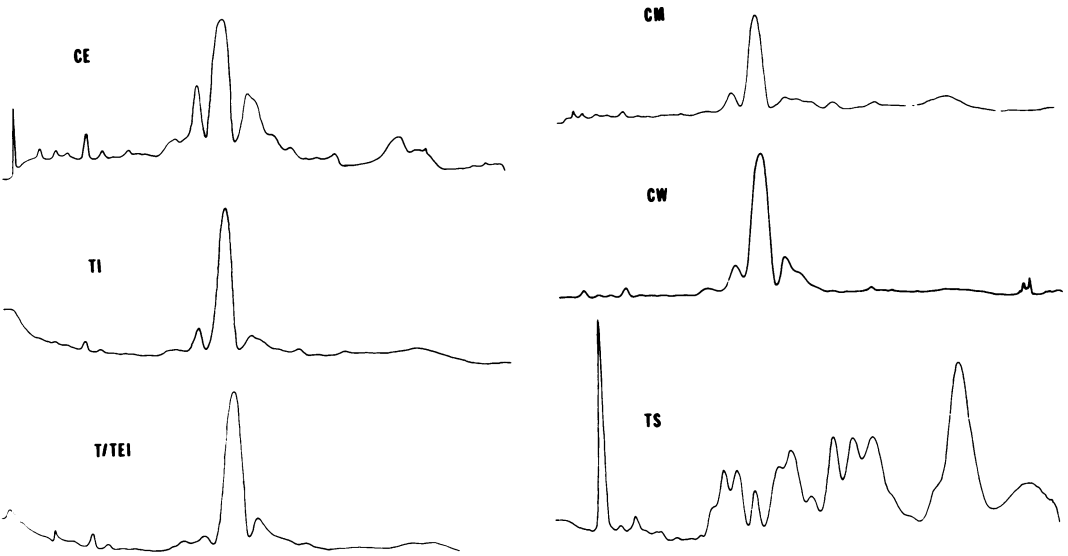

FIG. 5. *Optical density scans of SDS-polyacrylamide gels of various cell envelope fractions. All gels stained with Coomassie blue and scanned at 550 nm.*

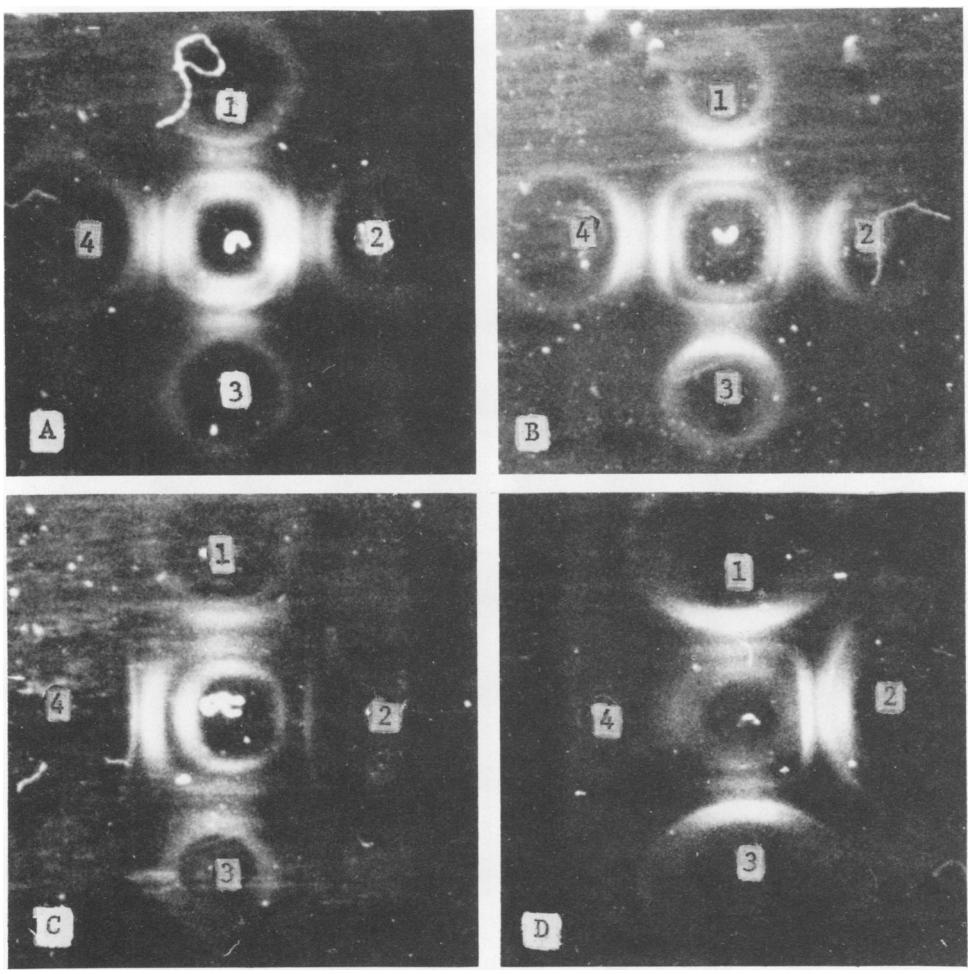

FIG. 6. *Immunodiffusion patterns of antisera to various cell envelope fractions against homologous and heterologous fractions and complete cell envelopes. All fractions adjusted to 0.75 mg of protein per ml and solubilized in 0.25% SDS. (A) CM antiserum versus: 1-CE, 2-CM, 3-CE, and 4-CW. (B) CW antiserum versus: 1-CE, 2-CM, 3-CE, and 4-CW. (C) TS antiserum versus: 1-CE, 2-T/TEI, 3-CE, and 4-TS. (D) T/TEI antiserum versus: 1-CE, 2-T/TEI, 3-CE, and 4-TS.*

two of the major precipitin lines obtained with both CM and CW sera represented the same antigen in all three fractions. Four to five precipitin lines are evident in the reaction of CM antiserum against the homologous antigen, with three to four lines occurring against the CW and CE fractions (Fig. 6A). A similar pattern can be seen in the case of the CW antiserum. Four lines occurred in the homologous reaction, and three lines occurred in the reactions with CM and CE fractions (Fig. 6B). These cross-reactions are not surprising in view of the results of Schnaitman (23), who estimated that similarly prepared fractions of *E. coli* contained approximately 10% cross-contaminating material.

A different picture was obtained with antisera to the fractions obtained by Triton extraction (Fig. 6C and D). Antiserum to the TS fraction reacted with the homologous antigen, producing three strong precipitin lines, at least one of which is seen in the reaction against CE, but the reaction was very weak against T/TEI (Fig. 6C). The T/TEI antiserum elicited two to three precipitin lines against the homologous antigen, one of the lines being in common with one formed with the CE fraction, but a very weak reaction or no reaction was formed against the TS fraction (Fig. 6D). It is also shown that TS and T/TEI antisera reacted strongly with CM and CW fractions (Fig. 7A and B). CM antiserum reacted strongly with the TI fraction, but

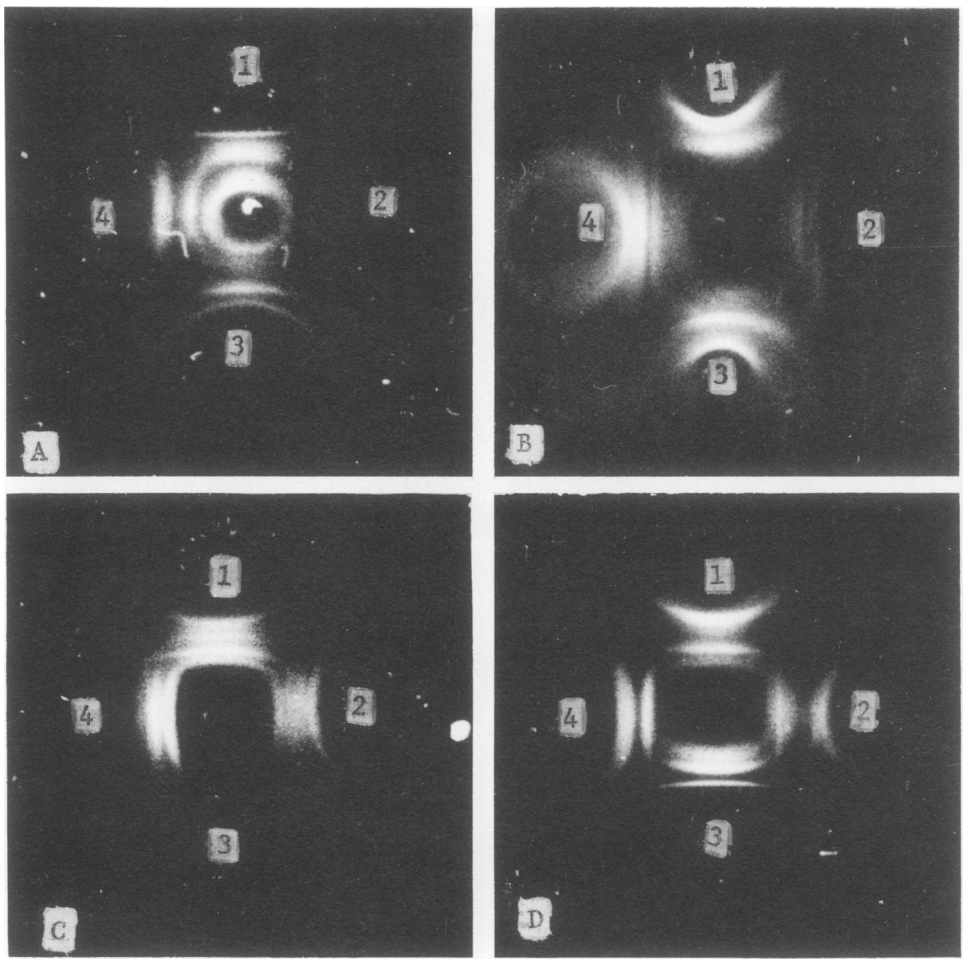

FIG. 7. *Immunodiffusion patterns of antisera to various cell envelope fractions against homologous and heterologous fractions. All fractions adjusted to 0.75 mg of protein per ml and solubilized in 0.25% SDS. (A) TS antiserum versus: 1-TS, 2-T/TEI, 3-CW, and 4-CM. (B) T/TEI antiserum versus: 1-T/TEI, 2-TS, 3-CM, and 4-CW. (C) CM antiserum versus: 1-CM, 2-CW, 3-T/TEI, and 4-TS. (D) CW antiserum versus: 1-CW, 2-CM, 3-TS, and 4-T/TEI.*

only weakly or not at all with the T/TEI fraction (Fig. 7C). Antiserum to CW, on the other hand, reacted with both TI and T/TEI fractions.

These results indicate that Triton extraction was more efficient in removing CM antigens from the CW fraction than density gradient centrifugation. The T/TEI fraction apparently contained negligible amounts of CM antigen, but the TS fraction contained both CM and CW antigens, as expected from the results with *E. coli* (23). The results also show that all fractions were capable of eliciting good precipitin antibody production in rabbits.

The killing effect of the rabbit antisera to the various CE fractions was determined against the homologous strain Nor-7. Table 1 shows the results of the bactericidal tests and indicates that all fractions were capable of eliciting bactericidal antibody production in rabbits. Prebleed sera from all rabbits were negative. In early experiments (results not shown), the antisera to CM and CW fractions showed no killing effect on a group C meningococcal strain, Nor-20. In addition, antiserum obtained from Naval Biomedical Research Laboratory, Oakland, Cal., prepared against whole cells of a group C strain (SD1C) of high bactericidal titer against the homologous organism had no effect against strain Nor-7. Bactericidal activity against a heterologous strain of group B meningococci was not determined.

Toxicity. Preliminary determinations of the toxicity of the various fractions were made by the Shwartzman reaction in rabbits. The results indicated that the TS fraction was the most toxic, the T/TEI fraction was the least toxic, and the TI and CW fractions were more toxic than the CM fraction. A more precise comparison of toxicity was carried out in rats sensitized with lead acetate. The combined results of several tests are shown in Table 2 and are evaluated statistically in Table 3. These tests included a preparation of LPS purified from the same strain (Nor-7) of group B meningococci. There appeared to be three distinct levels of toxicity, those of the LPS, of the four fractions CE, CW, CM, and TI, and of T/TEI. The mean lethal dose of the LPS of meningococci is quite small, indicating high toxicity. It is smaller, for example, than the mean lethal dose of LPS derived from *Serratia marcescens* (11) or *Pseudomonas aeruginosa* (1), but about as toxic for sensitized rats as the LPS of *E. coli* (L. A. Kiesow, personal communication). The mean lethal dose of the four fractions of intermediate toxicity was greater than that of the LPS by about two orders of magnitude. A difference in toxicity between CW and CM, suggested by the Shwartzman reaction, was not seen in experiments with sensitized rats, possibly because of the wide difference in susceptibility of the individual rats. The T/TEI fraction seemed to have a level of toxicity about one order of magnitude smaller than the other CE fractions. If the assumption is made that toxicity reflects LPS content and the geometric mean is used for comparison, it appears that four fractions have approximately the same LPS content, which is somewhat less than 1% of their dry weight, and T/TEI fraction has slightly less than 0.1% LPS.

TABLE 1. *Bactericidal titers of rabbit antisera to cell envelope fractions*

Antisera[a]	Bactericidal titer against homologous strain[b]
Anti-CE	1:3,200[c]
Anti-CW	1:6,400
Anti-TS	1:12,800
Anti-T/TEI	1:12,800

[a] Three rabbit sera were pooled in each case.
[b] Strain Nor-7, group B, *N. meningitidis*.
[c] Serum dilution killing more than 90% of the organisms compared with normal rabbit serum controls.

Conclusion. The described results clearly indicate that the procedures of Schnaitman (20, 22, 23) are directly applicable to the meningococci. The fractions obtained were entirely comparable to those of *E. coli* with the possible exception that Triton X-100 and Triton-EDTA extracting greatly reduced the rigidity of the cell wall layers. In contrast to those of *E. coli*, the TI and T/TEI fractions were small rounded vesicles. To what extent these results reflect biological differences between the two organisms or unrecognized variations in the extraction procedures is not known. Most significant is the confirmation by entirely different procedures—solubilization of labeled LPS in the case of *E. coli*, loss of toxicity for lead-sensitized rats of the insoluble fraction by *N. meningitidis*—that Triton-EDTA removes most of the LPS.

The results obtained may have practical applications. Wyle et al. (27) have conclusively shown that man does not respond to group B meningococcal polysaccharide antigens. CE proteins are therefore the most likely candidates for inclusion in a vaccine, but as it is noted in this paper these fractions are relatively toxic. The isolation of one fraction, T/TEI, of low toxicity (Tables 2 and 3) is encouraging. The ability of this fraction to elicit bactericidal antibody (Table 1) is significant in view of the

TABLE 2. *Lethality of various cell envelope fractions of N. meningitidis for rats sensitized with lead acetate*[a]

Dose (μg)	Lethality[b] of fraction					
	LPS	CE	CW	CM	TS	T/TEI
0.0025	0/10					
0.0050	3/10					
0.0075	1/10					
0.01	7/10					
0.05		0/10	0/10	0/10	0/10	
0.1	9/10					
0.5				0/10		
1.0	9/10	1/10	1/10	1/10	1/10	
1.25		1/10		7/20	2/10	
2.5		8/10	3/10	17/20	9/10	
5.0		7/10	6/10	15/20	7/10	0/10
7.5		10/10				3/10
10	9/10	9/10	10/10	9/10	10/10	2/10
25						5/10
50						9/10
100						10/10

[a] Rats weighing 180 to 220 g were injected intravenously with 10 mg of lead acetate dissolved in 0.5 ml deionized water and immediately thereafter with 1 ml of fraction, as indicated.

[b] Dead/total.

TABLE 3. *Statistical analysis of data presented in Table 2*

Fraction	LD_{50}[a] (μg)			LPS based on geometric mean (%)
	Low 95% fiducial limit	Geometric mean	High 95% fiducial limit	
LPS	0.003	0.016	0.084	(100)
CE	1.41	2.71	4.11	0.59
CW	1.92	3.02	4.76	0.53
CM	0.91	2.20	5.34	0.73
TS	0.35	1.88	10.2	0.85
T/TEI	11.6	19.3	32.1	0.08

[a] Mean lethal dose.

demonstration by Goldschneider et al. (8) of the importance of bactericidal antibody in human resistance to meningococcal infection.

It was shown, however, by Gold and Wyle (7) that the bactericidal antibody to group C meningococci is type-specific. Frasch and Chapman (3) have subdivided group B meningococci on the basis of bactericidal tests into several types and have demonstrated that the antigens responsible for these reactions are proteins (4). Although the immunological specificity of the T/TEI antigen has not been investigated, it is most likely that it too is type-specific. Thus, if further studies of this fraction indicate that it has immunogenic potential for man, an effective vaccine for group B meningococci may require the T/TEI fractions of more than one strain. However, Frasch and Chapman (5) have shown that almost 75% of the case isolates belong to serotype 2, the same serotype as the strain (Nor-7) used in this study.

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