PURIFICATION AND CHARACTERIZATION OF EIGHT CLASS 5 OUTER MEMBRANE PROTEIN VARIANTS FROM A CLONE OF *NEISSERIA MENINGITIDIS* SEROGROUP A

By MARK ACHTMAN,* MICHAEL NEIBERT,* BRIAN A. CROWE,* WOLFGANG STRITTMATTER,* BARICA KUSECEK,* EBERHARDT WEYSE,* MICHAEL J. WALSH,* BARBARA SLAWIG,* GIOVANNA MORELLI,* ALBRECHT MOLL,‡ AND MILAN BLAKE§

*From the *Max-Planck Institut für Molekulare Genetik, D-1000 Berlin 33; ‡Henning Berlin, D-1000 Berlin 42, Federal Republic of Germany; and the §Department of Microbiology, The Rockefeller University, New York, New York, 10021*

Neisseria meningitidis isolates from patients suffering from meningitis and from healthy carriers were obtained during (1982–1983) (1), and after (1984–1985) an epidemic of meningococcal meningitis in the Gambia, West Africa. This epidemic was part of the most recent epidemic wave of meningococcal meningitis in West Africa and is being analyzed in great detail with a variety of approaches in an attempt to understand the basic mechanisms responsible for epidemic bacterial disease. Representative serogroup A isolates from the Gambia all belonged to the clone A IV-1 (reference 2; Crowe, B. A., R. A. Wall, B. Kusecek, B. Neumann, T. Olyhoek, H. Abdillahi, J. Poolman, M. Hassan-King, B. M. Greenwood, and M. Achtman, manuscript in preparation), which was also responsible for concurrent epidemics in other West African countries and which had been isolated from patients in West Africa since the early 1960s (2). The Gambian bacteria were so similar that they may be regarded as variants of a single strain, yet they differed in the expression of certain outer membrane components. Many strains differed in the expression of the Class 5 and pilus proteins similar to data published by others (3, 4) for limited series of isolates from endemic cases of meningococcal meningitis; the significance of such variation remains unclear. For the Gambian epidemic, experiments are in progress to measure the development of protective antibodies, including those against Class 5 proteins, in humans upon carriage and after convalescence. Preliminary unpublished experiments revealed that the bactericidal activity of many human sera seems to be specific for the class 5 proteins but that such antibodies could not be revealed by Western blotting. Thus, to measure such antibodies, we decided to purify representative class 5 proteins to homogeneity from the Gambian isolates as well as to subject the purified proteins to a chemical and serological analysis. Before this work, the extent of our

This manuscript is dedicated to Thomas A. Trautner, whose continuous support and encouragement since 1972 have so facilitated the work presented here and elsewhere.

The work was supported in part by grants AC 36/4 from the Deutsche Forschungsgemeinschaft and AI-10615 from the National Institutes of Health. Dr. Blake is the recipient of an Irma T. Hirschl Award.

knowledge of meningococcal class 5 proteins was based on analytical experiments, DNA analyses, and extrapolation from information gained with gonococci.

The class 5 proteins of *N. meningitidis* are a group of heat-modifiable outer membrane proteins that contain several common proteolytic peptides and that migrate (after denaturation) on SDS-PAGE with an apparent size of ~28 kD (3, 5). The class 5 proteins have been concluded (6, 7) to correspond to the P.II proteins (8) of the *Neisseria gonorrhoea* outer membrane that also display these properties. Different purification schemes for P.II proteins have been described (9–12). The NH_2-terminal amino acid sequences of eight purified P.II proteins were uniform at numerous positions (10, 12) and were consistent with the NH_2-terminal DNA sequences of two *N. gonorrhoea opaE* genes (13). Strong hybridization has been observed between *opaE* DNA probes and meningococcal chromosomal DNA (7, 13, 14), and DNA sequencing has also shown strong conservation of parts of the sequence of gonococcal *opa* genes and the corresponding meningococcal *opr* genes (7). Initial experiments using published purification schemes failed with the meningococcal strains used. In this report, we present the purification methods for, and the characterization of, eight distinct class 5 proteins from clone IV-1 of *N. meningitidis* serogroup A.

Materials and Methods

Chemicals. All reagents not otherwise described were of p. a. quality from E. Merck (Darmstadt, FRG). Zwittergent 3-14 (Zw 3-14)[1] was from Calbiochem-Behring Corp. (La Jolla, CA). Empigen BB was from Albright & Wilson Ltd., Detergents Sector, Marchon Works, Whitehaven, Cumbria, England. SDS was from BDH Chemicals Ltd. (Poole, England; No. 44244, specially pure). 2,3-Dimercaptopropanol, 5-bromo-4-chloro-3-indolyl phosphate, EtBr, PMSF, Trizma hydrochloride, and Trizma base were from Sigma Chemical Co. (St. Louis, MO). Urea (ultra pure enzyme grade) was from Bethesda Research Laboratories (Gaithersburg, MD), acrylamide (No. 5521) was from Eastman Kodak Co. (Rochester, NY), and BIS (*N,N'*-methylene-bis-acrylamide) was from Bio-Rad Laboratories (Richmond, CA). TEMED (*N,N,N',N'*-tetramethylethylenediamine), nitro blue tetrazolium chloride (NBT), calf thymus DNA, Tween 20, Servalyt 3-10, Coomassie brilliant blue R-250 (Serva Blue R), and Coomassie brilliant blue G-250 (Serva Blue G) were from Serva Feinbiochemica (Heidelberg, FRG). PBE 118, Sephacryl S-200 (super fine), and Superose 12 (prep grade) were from Pharmacia Fine Chemicals (Uppsala, Sweden). Proteinase K (161 519) and *p*-nitrophenyl phosphate were from Boehringer Mannheim GmbH (Mannheim, FRG).

Buffers. TEN: 50mM Trizma, pH 8.0, 200 mM NaCl, 10 mM EDTA. PBS (per liter distilled water): 8 g NaCl, 0.2 g KCl, 1.44 g $Na_2HPO_4 \cdot 2\ H_2O$, 0.2 g KH_2PO_4, 2.9 g $Na_2HPO_2 \cdot 2\ H_2O$. Gey's basic salts solution (GBSS) contained per liter distilled water 8 g NaCl, 0.2 g KCl, 1.44 g $Na_2HPO_4 \cdot 2\ H_2O$, 0.2 g KH_2PO_4, and 1 g gelatin. After autoclaving and just before use, 1.8 ml of 0.5 M $CaCl_2$ and 0.5 ml of 1 M $MgCl_2$ were added. Carbonate buffer: 18 mM Na_2CO_3, 45 mM $NaHCO_3$. SDS-PAGE sample and electrode buffers were according to Laemmli (15), except that the sample buffer contained only 1% SDS (wt/vol) and the electrode buffer contained 0.15% SDS. Transfer buffer for Western blots consisted of 25 mM Tris, 200 mM glycine.

Bacteria. The bacteria used are listed in Table I. All were *N. meningitidis*, serogroup A, clone IV-1 (2, Crowe, B. A., et al., manuscript in preparation). Bacterial strains derived from a single colony were stored on multiple glass beads in skim milk at −70°C (16) and grown on supplemented GC agar plates (2) at 37°C, 5% CO_2, 70% relative humidity. A single glass bead was used as the inoculum for an agar plate and after overnight growth, the bacteria were subcultured to 75 agar plates (square format; 12 × 12 cm; 50 ml). These plates were

[1] *Abbreviations used in this paper:* GBSS, Gey's basic salts solution; NBT, Nitro blue tetrazolium chloride; TEN, tris, EDTA, NaCl buffer; Zw 3-14, Zwittergent 3-14.

TABLE I
List of Bacterial Strains and Sources

Purified protein	Strain used	Class 5 proteins expressed	Source
a	C962	a	Carrier, Gambia, 1984
b	C396	b,c	Patient, Gambia, 1983
c	C801	a,c,d	Carrier, Gambia, 1983
C	C911	C,d	Carrier, Gambia, 1984
d	C908	d	Carrier, Gambia, 1984
e	C922	e	Carrier, Gambia, 1984
f,g	B213	f,g	Patient, Ghana, 1973
h	B362	C,h	Patient, Cameroon, 1966

incubated for 16 h and the 40 g wet weight of bacterial growth was scraped together with a glass slide and resuspended with 8 ml of buffer (see below).

Sera. Rabbits were immunized intravenously with live bacterial preparations according to a protocol patterned on that of Zollinger and Mandrell (17): the rabbits were immunized on days 0, 17, and 33 with 2×10^8, 4×10^8, and 8×10^8 cells, respectively, and were bled on day 55. The rabbit sera were incubated at 56°C for 30 min to inactivate complement. For absorption experiments, stationary phase bacteria grown in supplemented GC broth were resuspended with an equal volume of GBSS and the sera were mixed with 0.4 volumes of this suspension. The mixture was rotated end over end for 16 h at 4°C. The bacteria were then removed by centrifugation and the adsorbed sera were stored in small portions at −70°C.

Alkaline phosphatase-conjugated affinity-purified rabbit Ig to mouse Ig from Dakopatts a/s (Copenhagen, Denmark) were used at a dilution of 1:500. Anti-rabbit IgG (whole molecule) alkaline phosphatase conjugate from Sigma Chemical Co. was used at a dilution of 1:1,000.

Hybridomas and mAbs. For the mAbs D309, 312.2C5.2, and B306, BALB/c mice were immunized subcutaneously with 5 μg of purified protein (a, C, or d, respectively) in CFA. Thereafter, preparations consisting of 1–2 μg of protein, half of which had been boiled for 5 min, plus 5 μg of purified LPS (either from a Gambian strain or from *Salmonella typhimurium*) were injected intraperitoneally on days 5, 8, 12, 15, and 20. On days 45 and 47, the mice were injected intraperitoneally with 2 μg of protein and on day 46 the mice were injected twice with 2 μg of protein mixed with 5 μg LPS. On day 49, spleen cells were fused with P3-X63-Ag 8.6.5.3 myeloma cells (18) using polyethylene glycol. Hybridoma cell lines were obtained by standard methods (19). Hybridomas synthesizing mAbs were recognized using the ELISA technique (see below) with microtiter plates coated with the purified protein used for immunization and the cell lines were cloned twice by limiting dilution. Antibodies were purified from culture supernatants using protein A-Sepharose chromatography.

For mAb 4B12/C11, mice were immunized intraperitoneally with 100 μg in IFA of a P.II protein purified from *N. gonorrhoea* R10 as previously described (10). At day 12, the mice were reinjected with 100 μg i.p. of the same protein in water. At day 24, the mice received a similar injection, and 3 d later the spleens were used for fusion as above. The hybridomas were screened for the production of crossreacting mAbs using the ELISA method with several different purified P.II proteins. The mAbs were purified using Na_2SO_4 and PEG precipitation.

Extraction Method. For extraction of class 5 proteins a, c, C, or d, the bacteria were suspended using an Ultra-Turrax (Janke & Kunkel, IKA Werk, Staufen, FRG) in 8 ml of 1 M sodium acetate, pH 4.0, containing 50 mM dimercaptopropanol. For extraction of proteins b, e, f, g, or h, the bacteria were suspended in the same buffer, except that the pH was 3.6. For all the proteins except class 5 b, 45 ml of 0.5 M $CaCl_2$ containing 5% (wt/vol) Zw 3-14 was added to the bacterial suspension. For class 5 b, Empigen BB was substituted for the Zw 3-14. The mixtures were dispersed for 3–4 min at room temperature with the Ultra-Turrax. Ice cold ethanol was added to a concentration of 20% (vol/vol) and the precipitated material was removed by centrifugation (Sorvall GSA rotor, 15,000 rpm, 10 min, 4°C). For extraction

TABLE II
Column Buffers Used in Chromatography

Columns	Class 5 proteins c,C	b,d,e,f,g,h	a
Mono Q	50 mM Trizma, pH 7.2	50 mM Trizma, pH 7.2	–*
PBE™ 118	–	–	25 mM TEA‡ pH 11.5
Mono S	50 mM Trizma, pH 7.2	50 mM acetate, pH 5.6	50 mM acetate pH 5.6
Superose™ 12	50 mM Trizma, pH 8.0, 200 mM NaCl		

All column buffers contained 10 mM EDTA and 0.5% (wt/vol) Zw 3-14.
* –, Column step not performed.
‡ TEA, triethylamine.

of the class 5 a protein, the pellet was suspended in 12 ml of 1 M ethanolamine, pH 10.6, containing 50 mM dimercaptopropanol, and then reextracted as above with 90 ml of $CaCl_2$/Zw 3-14 and treated with 20% ethanol. The pellets of the 20% ethanol precipitations were discarded and the supernatants were adjusted to 80% ethanol and incubated 1 h at 0°C.

After centrifugation of the 80% ethanol precipitate, the sediment (4 g wet weight) was resuspended with a syringe in 200 ml of the first column buffer listed in Table II but containing 5% Zw 3-14. This suspension was stirred at 20°C for 10 min. PMSF (dissolved in ethanol) was added to a concentration of 1 mM. The mixture was then stirred at 65°C for 15 min and cooled to 30°C. After centrifugation, the supernatant was filtered through a GVWP filter (Millipore Corp., Bedford, MA) and degassed.

Purification Procedures. All the following column operations were conducted at room temperature using a flow rate of 4 ml/min and the buffers are listed in Table II. Ion-exchange chromatography was performed using the 8 ml Mono S HR 10/10 and Mono Q HR 10/10 columns and an FPLC system (Pharmacia Fine Chemicals). Chromatofocussing was performed using a 175-ml column (column size 2.5 cm diameter × 50 cm long) packed with PBE 118.

For class 5 protein a, the material resuspended after 80% ethanol precipitation was subjected to chromatofocussing and the flowthrough fraction was precipitated overnight at 4°C with 80% ethanol, resuspended in 80 ml of the pH 5.6 column buffer containing 1% Zw 3-14, and applied to Mono S chromatography. For class 5 proteins c and C, the resuspended material was applied to the Mono Q and Mono S columns operating in tandem at pH 7.2 (Mono Q to Mono S). After the OD_{280} had returned to the baseline level, the Mono S column was detached from the Mono Q column and eluted at pH 7.2 as described below. For the other class 5 proteins, the resuspended material was applied to the Mono Q column (pH 7.2) and the flowthrough fraction was precipitated and resuspended in the pH 5.6 buffer as above before chromatography on the Mono S column.

The material that bound to the Mono S column was eluted with an NaCl gradient as shown in Fig. 1 *A*. The individual proteins eluted under the chosen conditions in one to three major peaks at a salt concentration between 75 mM and 160 mM. Fractions from each peak were pooled, samples were tested by SDS-PAGE, and peaks containing predominantly the desired class 5 protein were pooled and precipitated overnight at 4°C in 80% ethanol. The precipitated material was resuspended in 2.5 ml of TEN containing 4% Zw 3-14. This preparation was centrifuged and the supernatant applied to preparative gel filtration.

Gel Filtration. Preparative gel filtration was performed at room temperature and at a flow rate of 0.25 ml/min (7 cm/h) with a 1.6 cm diameter × 50 cm length (Pharmacia HR 16/50) column packed with 100 ml of Superose 12. Typical results are shown in Fig. 1 *B*. The yields of representative runs are presented in Table III. Analytical gel filtration was performed at a flow rate of 0.2 ml/min (15 cm/h) using 50-μl samples containing 100 μg of protein and a 1 cm diameter × 50 cm length column packed with 32 ml of Sephacryl S-200.

Calibration of the columns was performed using the same buffer system with and without Zw 3-14. The molecular weight markers used were Dextran Blue (2,000,000), β-amylase

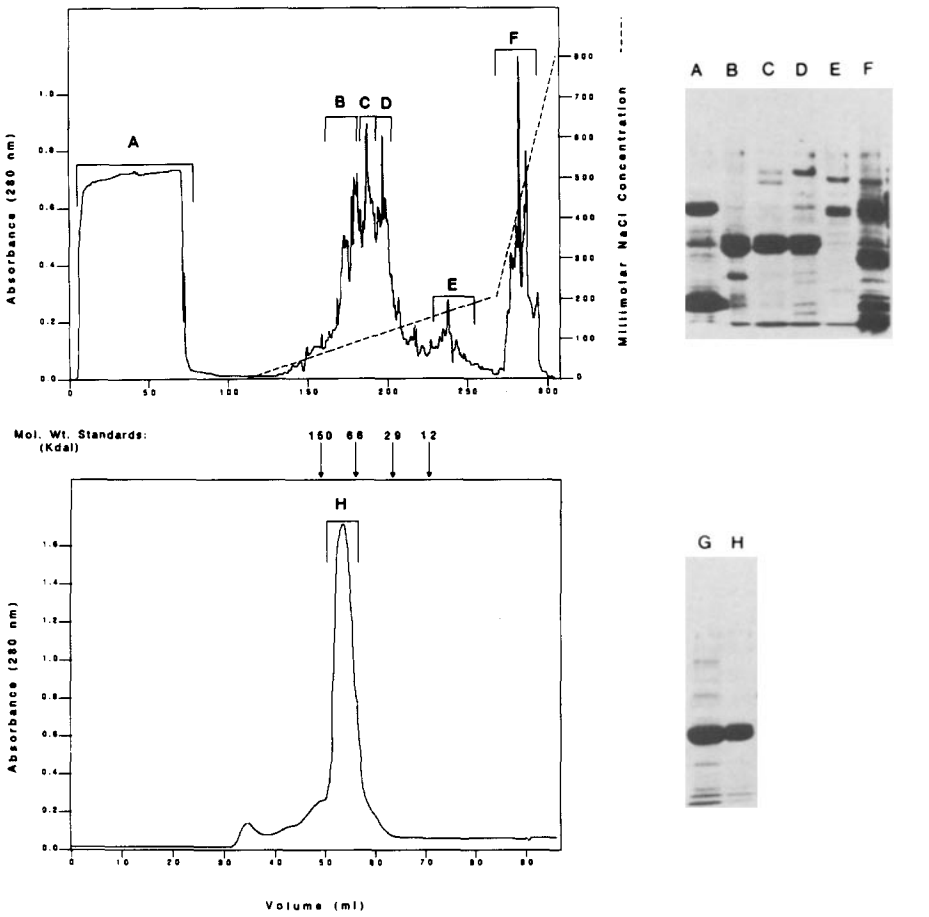

FIGURE 1. Purification of class 5 protein a. (*A*) Elution profile of the Mono S column. Fractions A–F were analyzed by SDS-PAGE with the results shown at the right. (*B*) Gel filtration on a Superose column. The loading material (fraction G, a pool of fractions B and C from part A) and the peak pooled in fraction H were analyzed by SDS-PAGE with the results shown at the right.

(200,000), alcohol dehydrogenase (150,000), bovine serum albumin (66,000), carbonic anhydrase (29,000), and cytochrome *c* (12,400).

SDS Gel Electrophoresis and Western Blotting. All protein samples that contained Zw 3-14 were first precipitated with 80% ethanol overnight at 4°C (8) before being resuspended in SDS-PAGE sample buffer. Native proteins were analyzed using these samples directly while denaturation was induced by boiling for 2 min. A 30:0.8 ratio for acrylamide/BIS was used to form stacking gels containing 0.125 M Tris, pH 6.8, 5% acrylamide and running gels containing 0.375 M Tris, pH 8.8, 11% acrylamide (15). Urea was added to the running gel mixture to a final concentration between 0 and 6 M, depending on the experimental design. Two sizes of slab gels were used: large (15 cm long × 16 cm wide) and small (6 cm long × 9.5 cm wide; Hoefer Mighty Small gels; Hoefer Scientific Instruments, San Francisco, CA). Both gel types were used with 0.75-mm spacers. Samples of <5 μl were applied to the stacking gel; electrophoresis was at 20 mA constant current for 4 h at room temperature for large gels and for ~1 h at 13°C for small gels. Thereafter, the gels were stained in 0.06% (wt/vol) Serva Blue G in 3.5% (vol/vol) perchloric acid overnight for large gels and 10 min for small gels. Destaining was in 5% (vol/vol) acetic acid for 8 h for large gels and for 1.5 h for small gels.

TABLE III

Yield and Properties of Class 5 Proteins

	Class 5 protein								
	a	b	c	C	d	e	f	g	h
Yield (mg/40 g wet weight):*	6	12	8	28	7	13	21	12	9
Gel filtration mol wt ($\times 10^{-3}$):	109	125	ND	111	113	117	123	117	118
SDS-PAGE mol wt (100°):	27.5	27.4	28.6	28.6	27.5	28.1	28.0	27.6	27.4
SDS-PAGE mol wt (30°):	22.9	22.9	28.2	28.2	22.8	22.7	23.8	23.1	22.3
pI:	8.8	9.0	8.9	8.9	8.9	9.1	8.75	9.3	9.0
Strongly immunogenic:‡	Yes	Yes	No	No	Yes	Yes	Yes	Yes	?
NH_2-terminal amino acid sequence:§	B	B	C	C	B	B	B	B	B
Epitope specificity:‖	a	b	c	c	b	b	?	b	?

* 80 g wet weight were used with double the yield presented here for proteins a and d.

‡ Proteins were considered strongly immunogenic when repeated intravenous injections of rabbits with live bacteria led to a strong response in western blotting against the corresponding class 5 protein. The results are summarized in the text.

§ The NH_2-terminal amino acid sequences referred to as B and C are shown in Fig. 4.

‖ The specificities are defined as follows: a, reaction with absorbed rabbit sera S2343 or S2373 or mAbs D309; b, reaction with absorbed sera S2352, S2377, S2337, S2361 or mAbs 312.2C5.2; c, reaction with rabbit serum S2435 or mAbs B306. Proteins with both a and b specificities react with mAbs 4B12/C11.

For boiled class 5 proteins, Western blotting was performed at 250 mA constant current in the TE 22 Transfer Unit (Hoefer Scientific Instruments) at 13°C for 1 h using small gels and nitrocellulose membranes (BA 85, Schleicher & Schuell, Dassel, FRG). Native proteins were transferred to Durapore membranes (GVHP, Millipore Corp.) according to a suggestion by Dr. E. C. Gotschlich. All nonspecified incubations were at room temperature. The membranes were blocked with 2% (wt/vol) BSA in PBS for at least 1 h, washed once in PBS containing 0.05% Tween 20, and incubated with hyperimmune sera or mAbs diluted 1:1,000 or more in PBS containing 1% BSA, 0.05% Tween 20. For the hyperimmune sera, the NaCl concentration in the buffers was increased to 650 mM to reduce non-specific sticking of Ig to class 5 proteins c and C. After 1.5 h shaking, the membranes were washed three times with PBS/Tween and then incubated with alkaline-phosphatase conjugated anti-antisera for 1.5 h. The membranes were washed five times and NBT and indoxyl phosphate were used at 37°C according to Blake et al. (20) to reveal the bound antibodies.

Analytical Methods. For all the following methods, the protein samples were ethanol precipitated before hydrolysis. Neutral sugars were liberated by hydrolysis in 4 M trifluoracetic acid at 100°C for 4 h and converted to alditol acetate derivatives (21) which were analyzed by gas chromatography using a gas chromatograph (3700; Varian Associates, Inc., Palo Alto, CA) and an SE 54 capillary column. 3-Hydroxy fatty acids were determined to quantitate LPS contamination. Fatty acids were released by transesterification in 2 N HCl/methanol at 85°C for 16 h. The resulting fatty acid methyl esters (22) were determined by gas-liquid chromatography using the Varian chromatograph and an OV 101 capillary column. Methyl heptadecanoate was used as internal standard.

Capsule measurements. Rocket gel electrophoresis was performed as described (23) using immunoglobulins prepared from an anti-serogroup A horse serum (supplied by Dr. John Robbins, National Institutes of Health, Bethesda, MD). Serogroup A capsule purified according to Gotschlich (24) was used as an internal standard.

LPS Measurements. LPS was analyzed using SDS-PAGE (15% acrylamide concentration in the running gel; 0.2% SDS in the electrode buffer) followed by silver nitrate staining using periodate according to Tsai and Frasch (25). LPS purified by the hot phenol water method described by Johnson and Perry (26) from a representative Gambian strain (C623) was used as an internal standard. The staining of material within the protein samples at the position of the LPS bands was visually compared with the staining of various amounts of purified LPS (sensitivity limit: 1 ng).

Nucleic Acid Measurements. Protein samples resuspended in TEN were treated with proteinase K (400 μg/ml) for 1 h at 60°C and EtBr was added to a concentration of 300 ng/ml. Calf thymus DNA treated with proteinase K was used as a standard and fluorescence was determined photographically.

Protein Determination. Protein was determined according to Schaffner and Weissman (27) using BSA in water as a standard.

Amino Acid Analysis. Samples (25 μg) of purified class 5 proteins were precipitated overnight at −30°C with ethanol (80% vol/vol) containing 0.02% (vol/vol) mercaptoethanol. After centrifugation, the protein pellet was washed with 5 ml of ethanol/mercaptoethanol, resuspended in 25 μl of 70% (vol/vol) formic acid, and centrifuged under vacuum till dry. For samples to be used for cysteine estimation, performic acid oxidization was performed in 25-μl volumes (28); the material was diluted with 250 μl of water and vacuum centrifuged till dry. All samples were resuspended in 100 μl of 5.7 N HCl containing 0.02 % (vol/vol) mercaptoethanol and 4% (vol/vol) thioglycolic acid and hydrolyzed at 110°C for 24 h in glass tubes under vacuum. Free amino acids were derivatized using ortho-phthaldialdehyde and were estimated using an automated HPLC-system (Waters Associates, Milford, MA) (29).

NH_2-Terminal Amino Acid Analysis. Purified Class 5 proteins (50 μg) were precipitated, washed, and resuspended in 50 μl of 70% (vol/vol) formic acid as above and applied to 1-cm diameter discs (GF/C glass fiber; Whatman Ltd., Maidstone, England). Proteins b and d were degraded in a gas phase sequencer constructed and operated as described (30). Proteins c, C, e, f, g, and h were sequenced using a pulsed liquid sequencer (model 477A; Applied Biosystems, Inc., Foster City, CA) equipped with the model 12.0 PTH-amino acid analyzer. Proteins sequenced with the gas phase instrument were applied to aminopropyl-derivatized glass fiber discs prepared as described (31) and proteins sequenced with the pulsed liquid instrument were applied to glass fiber discs that had previously been precycled with 1 mg of Polybrene (Biobrene; Applied Biosystems, Inc.).

Isoelectric Focusing. 0.2-mm acrylamide gels (3% T, 4% C) containing 3% Servalyt 3-10 were cast on GelBond PAG film (Pharmacia Fine Chemicals). Protein samples were solubilized in 5% Zw 3-14, 0.1 M glycine and applied to slots precast at positions corresponding to a neutral pH. Focusing was performed for 4,000 Volt-hours at 12°C using Anode Fluid 3 and Cathode Fluid 10 (Serva Feinbiochemica). The gels were fixed in 20% TCA, stained in Coomassie brilliant blue R-250 in methanol/acetic acid/water (40:10:50 vol/vol) and destained in the latter solvent.

ELISA. Protein samples in 0.5% Zw 3-14 were diluted in 0.06 M carbonate, pH 9.6, to a concentration of 2 μg/ml, and 50-μl aliquots were used (overnight at 4°C) to coat the wells of polystyrene plates (Linbro/Titertek, Flow Laboratories, Inc., McLean, Virginia). The following incubations were at room temperature. The wells were washed with PBS, blocked with 200 μl amounts of 10% FCS in PBS for 1 h and washed with PBS containing 0.05% Tween 20. Mouse or rabbit antibodies diluted in PBS containing 2% FCS and 0.05% Tween 20 were allowed to react for 1 h. The wells were washed with PBS/Tween and alkaline phosphatase-conjugated anti-antibodies diluted in PBS/FCS/Tween were allowed to react for 1 h. After washing in PBS/Tween, *p*-nitrophenyl phosphate substrate in diethanolamine buffer (32) was added. The reaction was stopped after 30 min at 37°C with 1 N NaOH and absorbance was measured at 405 nm.

Results

Criteria for Distinguishing and Naming Class 5 Variants. Membrane preparations from 788 subcolonies of 317 *N. meningitidis* serogroup A isolates from the Gambian epidemic were analyzed by SDS-PAGE (Crowe, B. A., et al., manuscript in preparation). The class 5 proteins varied but the variation seemed to be limited. The individual proteins expressed by any one subcolony seemed to fall into only six categories on the basis of their electrophoretic mobility and concentration and were assigned the designations a, b, c, C, d, or e. Proteins a, b, d, and e migrated at distinct characteristic positions regardless of urea concentration, while the migration of proteins

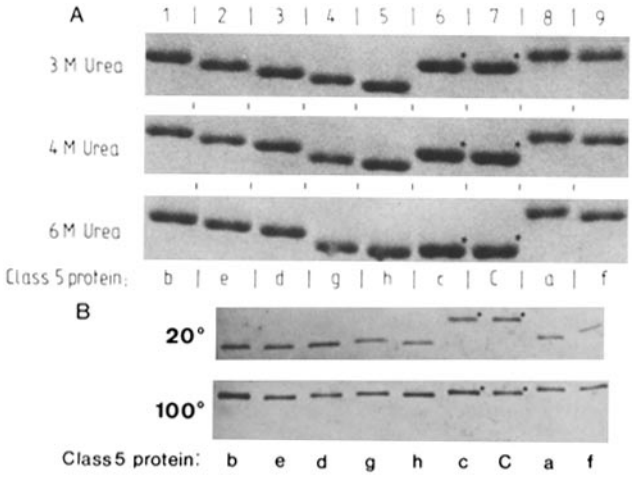

FIGURE 2. Migration of class 5 proteins at different urea concentrations. (*A*) 500 ng samples of the purified proteins were boiled in sample buffer and analyzed with SDS-PAGE using large gels containing 3, 4, or 6 M urea in the running gel. Only that part of the gel containing the class 5 proteins is shown in order to show the relatively minor differences in migration. The class 5 c and C proteins are marked with a star to emphasize that it is primarily these proteins that migrate differentially at different urea concentrations. (*B*) Boiled and unboiled samples were analyzed on small gels in the absence of urea. Other details were the same as for panel *A*.

c and C was dependent on the urea concentration (Fig. 2). Proteins c and C were distinguished from the other proteins by analyzing all the membrane samples in duplicate with gels containing 3 or 4 M urea. The distinction between proteins c and C was the amount detected upon SDS-PAGE of the membrane preparations: C designates a protein present in large amounts that when present in small amounts was designated c. In contrast, when present, a, b, d, or e were detected in comparable amounts within the membranes of different strains. The proteins synthesized by any one strain seemed to be associated randomly (Crowe, B. A., et al., manuscript in preparation).

Class 5 proteins vary dramatically between diverse meningococci in regard to their electrophoretic mobility. However, it seemed possible that within genetically closely related meningococci, the number of class 5 protein variants would also be limited. Therefore, 60 other clone A IV-1 strains isolated from West Africa between 1963 and 1983 (2) were screened and three further electrophoretic variants of the class 5 proteins, designated f, g, and h, were found. Proteins g and h differed in migration from a, b, c, C, d, and e at all urea concentrations, whereas protein f could only be distinguished from a and from c/C by using different urea concentrations (Fig. 2).

Eight strains were chosen from the meningococci screened that posessed few class 5 protein variants (Table I) for purification of these different variants.

Purification Methods. Initial attempts to purify the proteins described above met with little success. Furthermore, as since reported for *N. gonorrhoea* P.II proteins (12), no one method could be used to purify all the class 5 proteins. Three methods were devised as modifications of the methods described by Blake and Gotschlich (10) and Baritt et al. (12), which succeeded for all the class 5 proteins (Fig. 3). These methods also have the advantage over the currently published methods in that they are both efficient and quick.

Bacteria were extracted in $CaCl_2$ with Zw 3-14 as described by Blake and Gotschlich (10), except that the pH conditions were adjusted to the class 5 protein in ques-

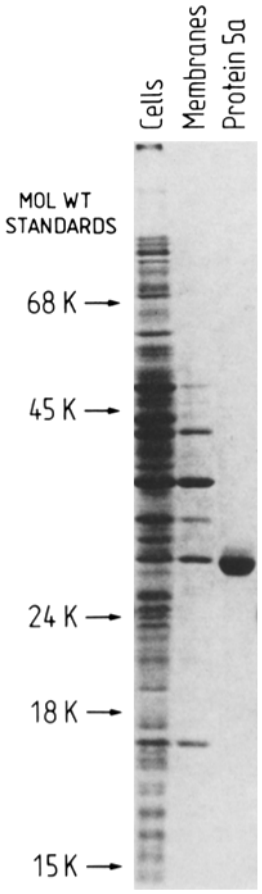

FIGURE 3. A comparison of whole cells, outer membranes, and purified protein by SDS-PAGE. Strain C962 cells and membranes are compared on a large gel with protein a purified from this strain after boiling samples in SDS.

tion. At pH values between 4.0 and 10.6, LPS is extracted as well as protein (10; data not shown), and therefore all extractions were performed outside this pH range. The extracted material was precipitated with 20% ethanol and the supernatant precipitated with 80% ethanol according to Blake and Gotschlich (10). Concentrations >3% proved to be necessary to resuspend the pellet after precipitation and routinely 5% Zw 3-14 was used. Efficient solubilization was only achieved with the meningococcal strains when the material was treated briefly at 65°C; under these conditions, the inhibition of protease activity with PMSF was also necessary. All subsequent steps were performed using an FPLC system because of the speed and efficiency of this chromatographic system. Finally, the concentration of Zw 3-14 used in running buffers was raised to 0.5% because lower concentrations were less effective.

The resuspended material was first applied to anion-exchange chromatography or chromatofocussing to remove unwanted components. Essentially all class 1, class 3, and class 4 proteins were removed at this step and the class 5 proteins did not bind to these columns. The class 5 proteins were then purified by cation-exchange chromatography at a pH suitable for the individual variant (either pH 5.6 or 7.2). The remaining contaminating LPS ran in the flowthrough fraction while a series

of sharp peaks containing predominantly class 5 protein were eluted at relatively low NaCl concentrations (Fig. 1 *A*). The jagged shape of the peaks suggests that different macromolecular complexes were being eluted and the SDS-PAGE analysis of individual fractions confirmed that each peak contained different contaminating proteins (Fig. 1 *A*). However, upon gel filtration, most of these contaminating complexes ran at higher apparent molecular weights and eluted before the pure class 5 protein (Fig. 1 *B*). The purification procedures are summarized in Table II. Using the methods described and the FPLC system, it was possible to purify 6–28 mg of individual class 5 proteins from 40 g wet weight of bacteria (Table III) within two working days. The methods have also been used to isolate these proteins from additional Gambian strains with no modifications and comparable results.

Purity. Nucleic acid contamination was ≤0.025% using fluorescence with EtBr and ≤0.1% based on neutral sugar analysis. LPS contamination ranged from 0.2% to ≤0.025% using SDS-PAGE plus silver nitrate staining and ≤0.1% by measuring LPS-specific 3-OH 14:0 and 3-OH 12:0 fatty acids. The c and C proteins, but not the other class 5 proteins, stained strongly with silver nitrate; this staining cannot be accounted for by residual fatty acid contamination. Capsule polysaccharide contamination ranged for individual preparations from 0.25% to ≤0.025% based on rocket gel electrophoresis. On SDS-PAGE no other major contaminating protein bands were visible. Silver nitrate staining of SDS-PAGE gels and Western blotting with hyperimmune sera directed against living whole cells revealed minor contamination with other proteins. Except for proteins f and h, which possessed minor amounts of proteins g and C, respectively (see below), no cross-contamination of one class 5 protein with another was detected.

Molecular Weight. As may be seen from Fig. 1 B, the class 5 proteins migrated during gel filtration with an apparent molecular weight much higher than the subunit molecular weight of 22,000–28,000. Analytical gel filtration confirmed this result (Table III). The micellar molecular weight for Zw 3-14 is approximately 30,000;[2] thus the apparent molecular weights (Table III) indicate that the class 5 proteins migrate as trimers or tetramers upon gel filtration.

In the absence of urea and for all the class 5 variants except c and C, the subunit molecular weights determined from SDS-PAGE were considerably higher for the denatured form (100°C in SDS for 2 min) than for the unboiled samples (Fig. 2, Table III). However, the mobility of unboiled c and C proteins increased with urea concentration such that the apparent molecular weight of these proteins at 6 M urea was 22,800 (unboiled) vs. 27,100 (boiled). The migration of the other proteins was only marginally affected by urea, except for proteins a and f for which the apparent molecular weight of the boiled forms increased slightly with urea concentration.

Other Properties. The isoelectric points determined in ultra-thin polyacrylamide gels were all between pH 8.8 and 9.3 (Table III).

Unique NH_2-terminal sequences were obtained for all proteins except C, which was heterogeneous at the NH_2-terminus consistent with the removal of the NH_2-terminal alanine from ~20% of the protein. The heterogeneity may reflect endopep-

[2] This value may be extrapolated from the data in reference 33. Furthermore, BSA apparently migrated within the Zw 3-14 micelles during gel filtration and migrated with a molecular weight ~30,000 larger than expected.

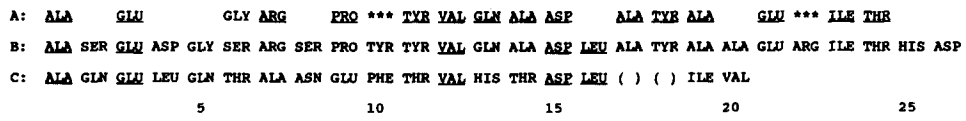

FIGURE 4. A comparison between different NH_2-terminal amino acid sequences of P.II and class 5 proteins. Amino acids common to all three sequences are underlined in *B* and *C* while amino acids common to sequences *A* and *B* are underlined in *A*. (*A*) The consensus sequence for *N. gonorrhoea* P.II proteins according to reference 12. Only the amino acids found in all gonococcal proteins are listed and the two insertions needed for alignment with the meningococcal sequences are indicated by ***. (*B*) A consensus sequence for meningococcal serogroup A class 5 proteins (length sequenced; discrepancies to the consensus sequence and/or uncertain assignments): a (24; no discrepancies), b (19; THR or SER at positions 6 and 8; ASP or HIS at position 15; ALA or ARG at position 19), d (20; ASP or THR at position 15), e (26; no discrepancies), f (10; no discrepancies), g (26; position 5 is SER), and h (10; no discrepancies). (*C*) Consensus sequence for class 5 proteins c and C. 12 amino acids were determined for c and 20 for C with no discrepancies. Positions 17 (probably LYS) and 18 (probably SER) were uncertain and are indicated by parentheses.

tidase activity, stuttering during synthesis, and/or may be a result of the isolation procedure. The primary response was accepted for the comparisons presented in Fig. 4. At positions 17 and 18 during the protein C sequencing, no signal was obtained but a strong signal resumed at cycle 19. Proteins a, b, d, e, f, g, and h were identical to the extent sequenced except for one amino acid substitution (SER for GLY at position 5) in protein g (Fig. 4). This common sequence was also identical for the first 25 amino acids to the sequence predicted from DNA sequencing of an *opr* gene for a meningococcal serogroup C class 5 protein and was identical except for position 5 to the sequence predicted from a second serogroup C *opr* gene (7). Furthermore, this sequence was congruent at 15 of 16 amino acids with the consensus sequence for gonococcal P.II proteins (12) if two single amino acid insertions (indicated by *** in Fig. 4) were included in the gonococcal consensus sequence. The sequences for proteins c and C were identical for the 12 amino acids sequenced in both proteins; the 20-amino acid sequence for protein C was only homologous at four positions to the adjusted gonococcal consensus sequence and differed at all other positions but one from the sequence for the other class 5 proteins.

Amino acid analyses showed that none of the proteins were identical, with the possible exception of c and C. These two proteins clearly differed in content of LEU and TYR (underlined in Table IV) from all other class 5 proteins.

Immunogenicity and Serological Specificity. 16 rabbits were injected intravenously with live bacteria containing various combinations of class 5 proteins. Sera from these animals were tested by Western blotting against boiled membrane preparations from bacteria containing the corresponding class 5 proteins and where a reaction against a class 5 protein was seen, it was one of the strongest reactions seen against any membrane protein (34). An attempt was made to obtain mono-specific sera from those yielding strong reactions by adsorption with bacteria differing from the immunogen only in class 5 protein content.

Six of seven rabbits immunized with Gambian bacteria containing class 5 a protein yielded sera which reacted strongly with the "a" protein in Western blots. Sera which were monospecific for the protein in Western blots and bactericidal tests were readily obtained from two of these sera.

TABLE IV

Amino Acid Analyses of the Purified Class 5 Proteins

Amino acid	a	b	c	C	d	e	f	g	h	V0	V28
Ala	23	20	15	14	21	19	20	25	19	15	15
Glx	17	19	23	23	18	17	20	17	32	10	16
Asx	28	28	25	25	19	22	25	22	22	25	30
Leu	12	14	**23**	**22**	12	14	10	14	16	12	13
Gly	23	20	21	21	23	24	16	33	25	22	20
Lys	16	19	28	26	27	19	17	26	15	16	17
Ser	13	11	12	12	14	16	16	12	14	21	15
Val	16	17	10	11	13	16	16	10	11	15	14
Arg	18	15	11	10	13	14	13	14	12	20	17
Thr	17	15	16	17	13	14	17	11	11	15	18
Ile	9	10	13	13	9	9	9	9	11	15	11
Met	1	1	0	0	1	2.5	1	1	3	2	0
Phe	7	9	10	11	9	9	9	7	8	8	9
Tyr	16	15	**8**	**8**	15	15	16	15	17	16	17
Cys	0	0	0	0.5	0.3	0	0	0	0	0	0
Trp	1	1	2	2.4	1	2	1	1	0	3	3
His	9	9	6	6	13	11	13	10	6	12	12

The molar ratios of each amino acid were calculated using a molecular weight of 25,000 for the Class 5 proteins. The data for the *N. gonorrhoea* V0 and V28 proteins were calculated from the sequences in reference 13.

Sera from the five rabbits immunized with bacteria expressing b, e (2 rabbits), ab, or abCd reacted strongly in Western blots with b, d, e, and g proteins but not with the a, c, C, f, or h proteins. Absorption of four of these sera with bacteria lacking b, d, and e yielded monospecific sera with the same specificities. The latter reacted in bactericidal tests with bacteria possessing b, d, or e and did not react with bacteria lacking these proteins but possessing proteins a and/or c/C. Bacteria expressing class 5 protein g were not tested.

None of the five rabbits immunized with bacteria containing c or C protein reacted strongly with the c or C protein in Western blots. A serum that reacted specifically in Western blots for c/C protein was obtained upon immunization of rabbits with pure C protein and CFA and IFA; this serum was not bactericidal.

Attempts to obtain monospecific sera from rabbits immunized with bacteria expressing Cf, fg, and Ch proteins failed.

Thus we conclude that the c/C proteins are poorly immunogenic in rabbits while the antigens associated with the class 5 proteins a, b, d, e, and g are among the primary epitopes on the cell surface. Furthermore, the results define three distinct serological specificities which are associated with the class 5 protein a, class 5 proteins c and C, and the class 5 proteins b, d, e and g. The serological specificity and immunogenicity of the class 5 proteins f and h remain undefined. These results are summarized in Table III.

Hybridoma cell lines have been isolated from mice immunized with purified a, c, or d proteins, which synthesize antibodies with the following specificities (mAb designation) in Western blots and ELISA tests against the purified proteins: α-a (mAb D309), α-c/C (mAb B306), and α-b,d,e,g (mAb 312.2C5.2) (Fig. 5). Broadly cross-reactive mAbs (mAb 4B12/C11) isolated against a gonococcal P.II protein were found

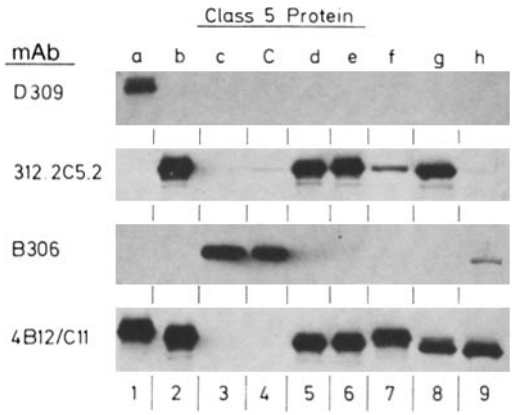

FIGURE 5. Western blot analysis of the purified class 5 proteins with different mAbs. The mAbs are indicated at the left, while the class 5 proteins are listed across the top. SDS-PAGE was performed on small gels containing 3 M urea and only the part of the gel containing the class 5 proteins is shown for clarity. The reaction of 312.2C5.2 in lane *7* reveals the minor contamination of protein f with protein g. Similarly, the reaction of B306 in lane *9* reveals the minor contamination of protein h with protein C.

to react in Western blots and ELISA tests against proteins a, b, d, e, f, g, and h but not c or C (Fig. 5). These mAbs are referred to below as α-a, α-c/C, α-b,d,e,g, and α-a,b,d,e,f,g,h, respectively. Similar reactions to that obtained with α-a,b,d,e,f,g,h were found with the broadly crossreacting mAbs 6D2/D9, 10A7/5E, 11B12/G9, and 5H4/F8 isolated in a similar manner to mAb 4B12/C11 by Milan Blake, The Rockefeller University, New York, NY.

Distribution of Class 5 Proteins. Our clonal analysis (2) had defined the genetic relationships within serogroup A and enabled a selection of strains known to represent the whole genetic diversity of that serogroup. We tested cell membranes from 83 such representatives by Western blotting with mAbs. The bacteria were chosen to represent clone I-1 which was responsible for one pandemic between 1967 and the mid-1970s, clone III-1 which was responsible for a second pandemic which began in the mid-1970s, and clone IV-1 which has been isolated from West Africa since the early 1960s. Furthermore, strains from the 34 distinct ETs defined within the four subgroups of serogroup A as well as older strains isolated between 1915 and 1950 (2) were also included. The results (Table V) showed that protein bands reacting with either α-a,b,d,e,f,g,h or α-c/C were found in each of the groups tested. Only few bands reacting with α-a were found but these were detected in all the bacterial

TABLE V

Western Blot Reactions of Diverse Serogroup A Strains with Anti-Class 5 mAbs

Bacterial group	Number of strains*	Number of protein bands which reacted with: 4B12/C11 α-a,b,d,e,f,g,h	B306 α-c,C	D309 α-a	312.2C5.2 α-b,d,e,g
Clone A I-1	17	19	11	0	0
Clone A III-1	14	13	5	2	0
Clone A IV-1	17	16	13	5	3
Strains from 1915–50	19	12	5	2	0
34 ETs	34	33	20	4	0
All strains	83	75	45	11	3

* The number of strains in each group was calculated separately and the groups overlap such that the total is >83.

groups tested except for ETs and clones belonging to subgroup A I. Only (three) clone A IV-1 bacteria reacted with α-b,d,e,g. The oldest strains found to react with each of these antibodies were from the years 1930 (α-a,b,d,e,f,g,h), 1937 (α-c/C), 1943 (α-a), and 1981 (α-b,d,e,g). All the bands reacting with α-c/C migrated identically under the SDS-PAGE conditions used, whereas striking differences were found between the electrophoretic migration of proteins that reacted with the other mAbs.

Analysis of the same membrane preparations after SDS-PAGE followed by Coomassie blue staining revealed that all the class 5 protein bands recognized by their intensity and characteristic electrophoretic migration reacted with α-a,b,d,e,f,g,h and/or α-c/C. For 30 strains only one stained protein band was seen that appeared to react in Western blot reactions with both these mAbs. Western blotting of unboiled preparations in the absence of urea revealed that the class 5 protein band was resolved into two or more distinct bands which reacted only with α-c/C or with α-a,b,d,e,f,g,h. The bands reacting with α-c/C migrated identically to unboiled Gambian c/C protein, whereas the bands reacting with α-a,b,d,e,f,g,h all migrated much faster under these conditions and also showed electrophoretic variation from strain to strain. We conclude that all class 5 proteins in serogroup A react exclusively with only one of these two mAbs and that the differentiation on the basis of urea between native C and the other native proteins represents a general phenomenon.

25 bacterial strains of various other meningococcal serogroups have been tested with the same mAbs. Gambian isolates of serogroups 29E (16 strains), Y (7 strains), W135 (1 strain), and I (1 strain) reacted with α-a,b,d,e,f,g,h whereas only bacteria of the serogroups 29E and W135 reacted with α-c/C. Serogroup 29E bacteria reacted with α-a and serogroup Y bacteria reacted weakly with this antibody. No reactions were found with the α-b,d,e,g antibody. All six gonococcal P.II variants (a–f) described by Barritt et al. (12) and eight other representative P.II proteins reacted with α-a,b,d,e,f,g,h and none reacted with α-c/C. Thus we conclude that the c/C and a,b,d,e,f,g,h specificities are common within meningococci of different serogroups but possibly the c/C specificity is lacking in gonococci.

Discussion

We were struck by the variability of the class 5 proteins during our initial investigations of an epidemic of meningococcal meningitis in the Gambia (Crowe, A. B., et al., manuscript in preparation). Bacteria isolated from different sites of one patient or from close contacts often varied in their class 5 composition, suggesting that these proteins might play an unknown role in the infectious process. However, the number of class 5 variants was limited and only five electrophoretic variants were distinguished within hundreds of isolates from cases and healthy carriers taken over a 4-yr period. Further investigations showed that a large proportion of the bactericidal antibodies in sera from healthy humans reacted specifically with the class 5 proteins but that these reactions were not detected in Western blots (34). In gonococci, the comparable P.II proteins have been implicated in adhesion phenomena (8) but the detailed function of any of these proteins remains unknown. Thus we decided to purify and characterize the class 5 proteins from Gambian and related serogroup A meningococci in order to use the purified proteins for antigenic and functional analyses. Preliminary results demonstrate that pairs of sera from healthy

Gambians who were colonized by serogroup A meningococci after the first serum sample, showed a dramatic rise in antibody levels against the purified class 5 proteins described here. The antibody rise corrrelated with the class 5 proteins present in the colonizing bacteria. It therefore seems likely that the methods presented here will allow us to achieve our initial goal of quantifying human serum antibodies directed against the class 5 proteins.

P.II proteins have been purified from *N. gonorrhoea* (9–12), but the published purification methods needed to be modified for each meningococcal class 5 protein before they yielded aceptable results. The purification schemes described here resulted in a set of methods that are quick, reproducible, and easy to perform, as well as yielding reasonably large amounts of pure protein (6–28 mg from 40 g bacterial wet weight). The high purity of the final preparations was demonstrated by a variety of independent methods, including tests for LPS contamination, and are adequate even for serological analyses. Furthermore, unlike most other schemes for purifying outer membrane proteins from Gram-negative bacteria, the end material had never been exposed to SDS and all our results until now indicate that the use of Zwittergent has maintained the native structure.

The class 5 proteins migrated upon gel filtration in Zw 3-14 buffer with an apparent molecular weight of 109,000–123,000. After subtracting the micellar molecular weight of Zw 3-14 (~30,000), this result suggests that the class 5 proteins were purified as a trimer or tetramer. The uncertainty arises because the native protein subunits migrate with an apparent molecular weight of 22,000–28,000 in SDS-PAGE, while boiling in SDS raises their apparent molecular weight to 27,500–28,600. Although it has been reported that a similar purification procedure yields P.II monomers from gonococci (10), the reevaluation of the micellar molecular weight of Zw 3-14 raises the question whether purified P.II proteins migrate as monomers or also migrate as trimers or tetramers. The possible relevance of a multimeric form for antigenicity or function of these proteins remains to be investigated.

Due to the existence of electrophoretic variants and considering the situation with gonococcal P.II proteins, it seemed likely that different antigenic specificities would be detected among the class 5 protein but that all the variants would be similar in their basic properties, genetic structure, and transcriptional regulation. In agreement, the amino acid compositions and pI of these proteins were similar (Tables III and IV). Except at amino acid position 5, the NH_2-terminal amino acid sequences of the variants a, b, d, e, f, g, and h (but not c or C) were identical to each other and to the NH_2-termini predicted from the DNA sequences of two serogroup C *opr* genes (13). Very recently (35), two other class 5 NH_2-terminal sequences have been published that differ at 4 or 5 of the first 21 amino acids from the sequence presented here. The congruent sequence calculated for 8 purified gonococcal P.II proteins and predicted from two sequenced gonococcal genes (10, 12, 13) agreed with the meningococcal NH_2-terminal sequences at 15 of 16 positions within the first 24 NH_2-terminal amino acids (Fig. 4), if two amino acid insertions were allowed in the congruent sequence.

The serogroup A class 5 proteins all migrated much more slowly with boiled samples than with unboiled samples during SDS-PAGE in the presence of urea. At least for class 5 variants a, b, and d, cells that have lost or gained these proteins are found at a frequency of several percent within any culture. Thus, the results were similar

to those found for gonococcal P.IIs (8). Within this biochemical context, the primary novelty is that only a limited number of the class 5 proteins were expressed within an epidemic situation and that these proteins could be reliably recognized by electrophoretic migration alone.

Despite numerous attempts, we were unable to produce antibodies specific for each of the electrophoretic variants purified here. The rabbit monospecific sera and mAbs that were obtained possessed the serological specificities a (protein a) or b (proteins b, d, e, and g). A large proportion of the antibodies stimulated against live bacteria during intravenous immunization of rabbits and mice was directed against these epitopes and the monospecific rabbit antibodies stimulated complement-mediated killing of the bacteria. Furthermore, a novel class 5 protein group consisting of the c/C variants, which possessed different antigenic properties from those just described, was also recognized.

Although the amino acid analyses of the class 5 c and C proteins were only marginally different from the other proteins, the NH_2-terminal sequence agreed at only 5 of the first 20 amino acids with the sequence of the other class 5 proteins and at 4 of these positions with the modified congruent sequence. The following phenomena were also unique to the c/C variants: the shift in mobility on SDS-PAGE when unboiled and boiled samples were compared only became apparent with these proteins when urea was present during electrophoresis. Only the class 5 c and C proteins reacted strongly with silver nitrate. Different from the other class 5 proteins and the P.II proteins, the amount of this protein class varied between different strains such that the c and C proteins seem to reflect the expression of the same protein at different efficiencies (large amounts of C vs. very small amounts of c). Changes from c to C or vice versa have also been observed in the laboratory at frequencies comparable to the on-off switch of the other class 5 proteins, whereas complete loss of c/C expression is a very rare event. Finally, the c and C proteins were not strongly immunogenic in mice or rabbits and we have not yet obtained strongly bactericidal antibodies from experimental animals.

These observations demonstrate that there are basically two different types of class 5 proteins within clone IV-1 of serogroup A, namely, c/C vs. the others (a, b, d, e, f, g, h). This conclusion is strongly supported by our description here of broadly crossreactive mAbs that also distinguish these two protein groups. Such broadly crossreactive mAbs directed against gonococcal P.II proteins were found to react in Western blots with all the other class 5 protein variants but not with c or C, while mAbs raised against the c/C protein did not react with any of the other proteins (Fig. 5). Although only the data for one mAb of each type have been presented in detail here, comparable results were found with four other broadly crossreacting anti-P.II mAbs, with one other anti-c/C mAb and with rabbit monospecific anti-c/C serum. These results could be interpreted as reflecting an epitope specificity of the anti-c/C antibodies. However, the two antibody specificities subdivided all class 5 proteins detected in 106 diverse representatives of serogroups A, 29E, and Y into proteins that reacted with one or the other. Thus, even if the anti-c/C antibodies recognize a specific epitope, that epitope has been conserved for >50 yr in a protein of constant electrophoretic migration, whose migration is similarly affected by urea and that appears in different strains in large or small amounts. Because P.II proteins reacting with the anti-c/C mAbs have not been detected, it remains open whether a comparable protein exists in gonococci. Furthermore, neither the biological function of

the c/C proteins nor of the other proteins have yet been determined so that the significance of these observations for infection remains unclear. However, despite the similarities at the DNA level, gonococci and meningococci have drastically different disease and organ specificities and it is conceivable that the class 5 proteins, and especially the c/C variants, play a role in the ability of meningococci to cause meningitis.

It might be argued that the c and C proteins are not class 5 protein variants at all but represent distinct proteins with a similar subunit molecular weight. However, all the class 5 proteins share 5 of the first 16 NH_2-terminal amino acids (Fig. 4), a relatively high pI, a trimer/tetramer subunit composition (Table III), comparable amino acid contents (Table IV), and hypervariability of expression. We conclude that the meningococcal class 5 proteins can be subdivided into two subclasses that seem to be regulated differently, have a different antigenic specificity and genetic basis, and that might differ in function for the bacterial cell. One of these two subclasses corresponds to the currently known P.II proteins in gonococci.

Summary

Methods published for the purification of P.II proteins from *Neisseria gonorrhoea* have been modified to allow the purification of class 5 proteins from *Neisseria meningitidis* serogroup A bacteria. The five class 5 protein electrophoretic variants detected within an epidemic in the Gambia (a, b, c, d, and e) and three other variants (f, g, and h) found within other isolates of the same clone in West Africa have been purified with yields of 6–28 mg. The NH_2-terminal amino acid sequence for variant c differs from those of the other class 5 proteins, whereas the latter are very similar to the sequence predicted for two class 5 proteins from DNA analyses of serogroup C meningococci and determined for 8 P.II proteins from gonococci. Numerous other regulatory, chemical, and serological differences were found between the c protein and the other class 5 proteins such that we recommend that the class 5 proteins be subdivided into two subclasses. mAbs have been isolated that distinguish between these two protein subclasses and Western blotting with these antibodies enabled us to conclude that both protein subclasses were found in bacteria isolated from different epidemics and pandemics of the last 50 yr.

The Gambian bacterial strains were obtained from Drs. Robert A. Wall, Brian M. Greenwood, and Musa Hassan-King of the Medical Research Council, Fajara, the Gambia. Vital information unpublished at the time on the properties of P.II proteins was obtained from Drs. Emil C. Gotschlich, The Rockefeller University, New York, and Janne G. Cannon, University of North Carolina at Chapel Hill, NC. Fatty acid analyses were performed in collaboration with Drs. Otto Holst, Uli Zähringer, and Helmut Brade, Forschungsinstitut Borstel, Federal Republic of Germany and the NH_2-terminal amino acid sequencing and amino acid analyses were performed in collaboration with Dr. Brigitte Wittmann-Liebold, Max-Planck Institut für molekulare Genetik, Berlin.

Received for publication 10 February 1988 and in revised form 2 May 1988.

References

1. Greenwood, B. M., A. M. Greenwood, A. K. Bradley, K. Williams, M. Hassan-King, F. C. Shenton, R. A. Wall, and R. J. Hayes. 1987. Factors influencing susceptibility to meningococcal disease during an epidemic in the Gambia, West Africa. *J. Infection.* 14:167.
2. Olyhoek, T., B. A. Crowe, and M. Achtman. 1987. The clonal population structure of

Neisseria meningitidis serotype A isolated from epidemics and pandemices between 1915 and 1983. *Rev. Infect. Dis.* 9:665.

3. Poolman, J. T., S. de Marie, and H. C. Zanen. 1980. Variability of low-molecular-weight, heat-modifiable outer membrane proteins of *Neisseria meningitidis*. *Infect Immun.* 30:642.
4. Tinsley, C. R., and J. E. Heckels. 1986. Variation in the expression of pili and outer membrane protein by *Neisseria miningitidis* during the course of meningococcal infection. *J. Gen. Microbiol.* 132:2483.
5. Tsai, C.-M., C. E. Frasch, and L. F. Mocca. 1981. Five structural classes of major outer membrane proteins in *Neisseria meningitidis*. *J. Bacteriol.* 146:69.
6. Frasch, C. E., W. D. Zollinger, and J. T. Poolman. 1985. Serotype antigens of *Neisseria meningitidis* and a proposed scheme for designation of serotypes. *Rev. Infect. Dis.* 7:504.
7. Stern, A., and T. F. Meyer. 1987. Common mechanism controlling phase and antigenic variation in pathogenic *Neisseriae*. *Mol. Microbiol.* 1:5.
8. Swanson, J. 1981. Surface-exposed protein antigens of the gonococcal outer membrane. *Infect. Immun.* 34:804.
9. Heckels, J. E. 1977. The surface properties of *Neisseria gonorrhoea*: isolation of the major components of the outer membrane. *J. Gen. Microbiol.* 99:333.
10. Blake, M. S., and E. C. Gotschlich. 1984. Purification and partial characterization of the opacity-associated proteins of *Neisseria gonorrhoea*. *J. Exp. Med.* 159:452.
11. Newhall, V. W. J., L. B. Mail, C. E. Wilde III, and R. B. Jones. 1985. Purification and antigenic relatedness of proteins II of *Neisseria gonorrhoea*. *Infect. Immun.* 49:576.
12. Barritt, D. S., R. S. Schwalbe, D. G. Klapper, and J. G. Cannon. 1987. Antigenic and structural differences among six proteins II expressed by a single strain of *Neisseria gonorrhoea*. *Infect. Immun.* 55:2026.
13. Stern, A., M. Brown, P. Nickel, and T. F. Meyer. 1986. Opacity genes in *Neisseria gonorrhoea*: control of phase and antigenic variation. *Cell.* 47:61.
14. Aho, E. L., G. L. Murphy, and J. G. Cannon. 1987. Distribution of specific DNA sequences among pathogenic and commensal *Neisseria* species. *Infect. Immun.* 55:1009.
15. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680.
16. Jones, D., P. A. Pell, and P. H. A. Sneath. 1984. Maintenance of bacteria on glass beads at −60°C to −76°C. *In* Maintenance of microorganisms. B. E. Kirsop and J. J. Snell, editors. Academic Press, Inc., London. 35–40.
17. 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.
18. Kearney, J. F., A. Radbruch, D. Liesegang, and K. Rajewsky. 1979. A new mouse myeloma cell line that has lost immunoglobulin expression but permits the construction of antibody secreting hybrid cell lines. *J. Immunol.* 123:1548.
19. Köhler, G., and C. Milstein. 1975. Continuous culture of fused cells secreting antibodies of predefined specificity. *Nature (Lond.).* 256:495.
20. Blake, M. S., K. H. Johnston, G. J. Russel-Jones, and E. C. Gotschlich. 1984. A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on western blots. *Anal. Biochem.* 136:175.
21. Sawardeker, J. S., J. H. Sloneker, and A. Jeanes. 1967. Quantitative determination of monosaccharides as their alditol acetates by gas-liquid chromatography. *Anal. Chem.* 37:1602.
22. Rietschel, E. T., S. Hase, M. T. King, J. Redmond, and V. Lehmann. 1977. Chemical structure of lipid A. *Microbiology (Wash. DC).* 177:262.
23. Kusecek, B., H. Wloch, A. Mercer, V. Vaisänen, G. Pluschke, T. Korhonen, and M.

Achtman. 1984. Lipopolysaccharide, capsule, and fimbriae as virulence factors among O1, O7, O16, O18, or O75 and K1, K5, or K100 *Escherichia coli*. *Infect. Immun.* 43:368.
24. Gotschlich, E. C. 1975. Development of polysaccharide vaccines for the prevention of meningococcal diseases. *Monogr. Allergy.* 9:245.
25. Tsai, C.-M., and C. E. Frasch. 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Anal. Biochem.* 119:115.
26. Johnson, K. G., and M. B. Berry. 1976. Improved techniques for the preparation of bacterial lipopolysaccharides. *Can. J. Microbiol.* 22:29.
27. Schaffner, W., and C. Weissmann. 1973. A rapid, sensitive, and specific method for the determination of protein in dilute solutions. *Anal. Biochem.* 56:502.
28. Hirs, C. H. W. 1967. Performic acid oxidation. *Methods Enzymol.* 11:197.
29. Ashman, K., and A. Bosserhoff. 1985. Amino acid analysis by high performance liquid chromatography and precolumn derivatisation. *In* Modern Methods in Protein Chemistry. Vol. 2. H. Tschesche, editor. Walter de Gruyter and Co., Berlin. 155–171.
30. Reimann, F., and B. Wittman-Liebold. 1986. Gas-phase sequencing of peptides and proteins. *In* Advanced Methods in Protein Microsequence Analysis. B. Wittman-Liebold, J. Salnikow, and V. A. Erdmann, editors. Springer-Verlag, Berlin. 118–125.
31. Aebersold, R. H., D. B. Teplow, L. E. Hood, and S. B. H. Kent. 1986. Electroblotting onto activated glass. *J. Biol. Chem.* 261:4229.
32. Voller, A., D. E. Bidwell, and A. Bartlett. 1979. The Enzyme-linked Immunosorbent Assay (ELISA). Dynatech Europe, Guernsey, England. 38–39.
33. Herrmann, K. W. 1966. Micellar properties of some zwitterionic surfactants. *J. Colloid Interface Sci.* 22:352.
34. Slawig, B. 1986. Antigen-spezifität von Serum-Antikörpern in Bezug auf die Zelloberfläche von *Neisseria meningitidis* der Serogruppe A. Freie Universität, Fachbereich Biologie, Berlin. 1–114.
35. Kawula, T. H., E. L. Aho, D. S. Barritt, D. G. Klapper, and J. G. Cannon. 1988. Reversible phase variation of expression of *Neisseria meningitidis* class 5 outer membrane proteins and their relationship to gonococcal proteins II. *Infect. Immun.* 56:380.