INFECTION AND IMMUNITY, Mar. 1989, p. 1005–1007
0019-9567/89/031005-03$02.00/0

Vol. 57, No. 3

Purification, Cyanogen Bromide Cleavage, and Amino Terminus Sequencing of Class 1 and Class 3 Outer Membrane Proteins of Meningococci

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Received 22 June 1988/Accepted 4 December 1988

Meningococcal class 1 and 3 outer membrane proteins (OMPs) were subjected to cyanogen bromide treatment. The class 3 OMP was found to be resistant to cyanogen bromide, while the class 1 OMP was cut into two main fragments of 25 and 17 kilodaltons. The N-terminal sequences were determined for class 1 and class 3 proteins, which exhibit similarities to one another and to OMP I of gonococci. The C-terminal class 1 OMP fragment bound the bactericidal monoclonal antibodies tested.

Meningococcal outer membrane proteins (OMPs) are currently being evaluated in vaccination field trials to prove their efficacy in preventing meningococcal diseases (2, 5, 16). These type of vaccines, however, are composed of a mixture of proteins and some residual lipooligosaccharides (LOS). Class 1, 2/3, and 4 OMPs are present in all these vaccines, but some vaccines also contain class 5 OMPs (5). Meningococci are rather heterogeneous with regard to class 1 and class 2/3 OMP composition (1, 6).

The drawbacks of such meningococcal OMP vaccines are as follows. (i) Nonprotective antigens are included. (ii) The protective antigens present induce type-specific bactericidal antibody activity. (iii) The formulation of the OMP vaccine does not allow the construction of multivalent OMP vaccines that are needed to induce broadly reactive bactericidal antibodies. (iv) Broadly protective vaccines are needed to allow the use of a meningococcal vaccine in the general infant vaccine programs.

It will be of help to define the epitopes that can induce or bind bactericidal antibodies and do not reveal a too high intra- or interstrain variability. When the bactericidal activities of members of a monoclonal antibody library were compared, it became clear that the class 1 OMPs and LOS induce and bind the strongest bactericidal antibodies against group B meningococci (10, 11). Immunization of mice with an OMP vaccine results in class 1 OMP-specific bactericidal activity (10). In an infant rat meningitis model, class 1 OMP- and LOS-specific monoclonal antibodies are protective and class 3 OMP-specific monoclonals are not (12, 13). Class 1 and 2/3 OMPs are hard to separate, however, and we undertook this study to tackle that problem.

Purification and cyanogen bromide (CNBr) treatment of meningococcal OMPs. The growth conditions of strain H44/76 (B:15:P1:16) have been described previously (14). Lyophilized bacteria were extracted with 0.5 M $CaCl_2$–1% (wt/vol) Zwittergent 3-14–0.14 M NaCl (pH 4.0). One hundred milliliters of buffer was used for 1 g of bacteria. After resuspension, the pH was brought to 6.0, and the suspension was extracted for 1 h at room temperature. Bacteria were removed by centrifugation (1 h, 3,000 × *g*, room temperature), and the supernatant was brought to 20% (vol/vol) ethanol concentration and centrifuged (30 min, 10,000 × *g*, room temperature).

The supernatant was concentrated by diafiltration in an Amicon Hollow Fiber System (H1P 30-43; cutoff, 30,000). Ethanol and $CaCl_2$ were replaced by 0.1 M sodium acetate–25 mM EDTA–0.05% Zwittergent 3-14 (pH 6.0) by five successive diafiltration cycles (14). The pH was brought to 4.0, 20% (vol/vol) ethanol was added and incubated for 30 min, and the solution was centrifuged (30 min, 10,000 × *g*). The supernatant now contained rather pure class 3 OMP. Residual LOS was removed by column chromatography.

The pellet contained the combined class 1/3/4 OMPs. This material was further depleted of LOS by DEAE-Sepharose and Sephacryl S300 chromatography (2, 14). Class 1 OMP was purified from sodium dodecyl sulfate (SDS) gels and treated with CNBr, and the two fragments were purified (15). The purified class 1, 3, and 1/3/4 OMP products were dissolved in 70% (vol/vol) formic acid containing a 10× overdose of CNBr and were treated this way for 16 h at room temperature.

The CNBr and formic acid were removed under a stream of N_2, and the pellet was suspended in 0.2 M Tris hydrochloride–6 M urea (pH 7.2). This material was fractionated over a TSK-G2000 column by using a high-performance liquid chromatography (HPLC) system (no. 450-52; LKB Instruments, Inc.) and a diode array detection system (no. 1040A; Hewlett-Packard Co.).

Figure 1 shows the purified class 1 and 3 OMPs, the purified class 1/3/4 OMP mixture, and the effect of CNBr treatment. Class 3 OMP was unaffected by CNBr. Class 1 OMP was cut into two fragments of 25 and 17 kilodaltons (kDa). The class 4 OMP also appears to have been cut by CNBr. By comparing lanes 1 and 4 of Fig. 1, it can be concluded that the 21- and 10-kDa fragments from lane 1 are likely candidates to be class 4 OMP CNBr fragments.

Figure 2 shows the purification by HPLC gel filtration of the CNBr fragments after treatment of a purified class 1/3/4 OMP mixture. The 25-, 17-, and 10-kDa fragments could be separated. For analytical purposes, a second HPLC step was included (Fig. 3, left panel, lane 3).

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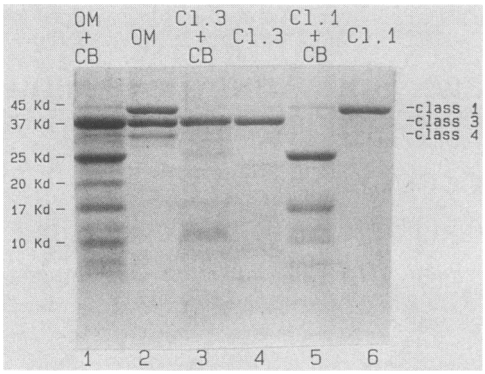

FIG. 1. SDS-polyacrylamide gel electrophoresis analysis of meningococcal OMPs (OM) and CNBr (CB) fragments. Lanes: 1, CNBr-treated class 1/3/4 OMP mixture; 2, class 1/3/4 OMP mixture; 3, CNBr-treated purified class 3 OMP; 4, purified class 3 OMP; 5, CNBr-treated purified class 1 OMP; 6, purified class 1 OMP. Kd, Kilodaltons.

the proteins and fragments were analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting by using monoclonal antibodies (15). The monoclonal antibodies used have been described before (9). Monoclonal antibody MN5C11G, P1.16 specific and bactericidal (13), bound to the 25-kDa class 1 OMP fragment as demonstrated in Fig. 3, right panel, lanes 2 and 3. Other P1.16-specific monoclonals showed similar binding activities.

Amino-terminal amino acid sequence analysis. Prior to sequencing, the protein or peptide solutions were subjected to four cycles of ice-cold trichloroacetic acid (7%) and acetone (80%) precipitation to remove any buffer (Tris, urea, etc.) and detergent (SDS, Zwittergent) components that would interfere with the Edman degradation chemistry. The resulting pellets were dissolved in 20% acetic acid, and approximately 500 nmol was immobilized on a Polybrene-coated glass fiber paper prior to the start of automated, repetitive Edman degradation with an Applied Biosystems model 477A pulsed liquid protein-peptide sequenator equipped with an on-line model 120A phenylthiohydantoin (PTH) analyzer. After each Edman degradation, the phenylthiazolinone derivative formed from each amino acid was converted to the more stable PTH derivative by treatment with 25% trifluoroacetic acid at 64°C for 20 min.

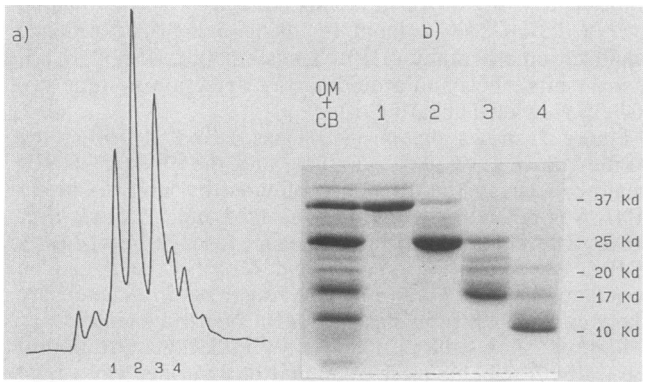

FIG. 2. HPLC purification of meningococcal OMPs (OM) and CNBr (CB) fragments by using TSK-G2000. (a) E_{280} of chromatogram. (b) SDS-polyacrylamide gel electrophoresis analysis of column fractions as indicated. Kd, Kilodaltons.

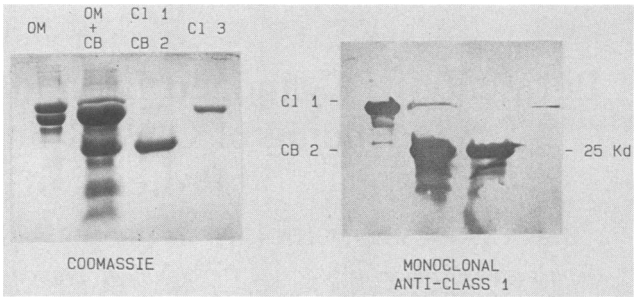

FIG. 3. Western blotting analysis of meningococcal OMPs and CNBr fragments by using monoclonal antibodies. Lanes OM and OM + CB are as described for Fig. 1. Results of protein stain (COOMASSIE) and with the monoclonal antibody Mn5C11G against class 1 OMP, subtype P1.16 (MONOCLONAL ANTI-CLASS 1), are shown.

The PTH derivatives were separated by reverse-phase HPLC over a Brownlee C-18 column (220 by 2.1 mm) with a two-solvent gradient system consisting of solvent A (5% tetrahydrofuran containing 27 ml of 3 M sodium acetate buffer [pH 3.8] per liter and 6.2 ml of 3 M sodium acetate buffer [pH 4.6] per liter) and solvent B (acetonitrile containing 500 nmol of oxidant scavenger, *N*,*N*-dimethyl-*N*-phenylthiourea [DMPTU] per liter). To improve chromatographic peak shapes and resolution of PTH-histidine and PTH-arginine, 0.5 ml of 12.5% trimethylamine was added to solvent A. Nominal HPLC parameters were as follows: flow rate, 200 μl/min; detector wavelength, 254 nm; and column temperature, 55°C. Optimal separation of PTH derivatives was achieved with the following linear gradient: 12% solvent B at time zero, 38% solvent B at 25 min, 90% solvent B at 25.1 min, 90% solvent B at 29 min. The PTH from each cycle was identified by comparison with a standard chromatogram of a mixture of PTH and amino acids (Applied Biosystems).

The amino-terminal sequences of class 1 and class 3 meningococcal OMPs are given in Fig. 4, together with the known amino-terminal sequences of gonococcal proteins I and meningococcal class 2 OMP (3, 4, 7). Interestingly, the N-terminal regions of meningococcal OMPs bear close homology until after residue 13. The two OMP sequences differ at positions 3 (Ser versus Thr) and 7 (Glu versus Thr) and become quite diverse after amino acid 13. The class 3 OMP

OMP	N-terminal sequence alignment
class 1, H44/76	D-V-S-L-Y-G-E-I-K-A-G-V-E-G-R-N-Y-Q-L-Q-L-T-E-A-Q-A
class 3, H44/76	D-V-T-L-Y-G-T-I-K-A-G-V-E-T-S-R-S-V-F
pI, 120176-2	D-V-T-L-Y-G-T-I-K-A
class 2, BNCV	D-V-S-L- -G-E-I-K-A-G
pI, R10	D-V-T-L-Y-G-A-I-K-A-G-V-Q-T-Y-R-S-V-E
pI, 33	V-T-L-Y-G-A-I-K-A
pI, FA19	D-V-T-L-Y-G-T-I-K-A-G-V-E-T-S-R-S-V-A
class 1,H44/76 C-terminal CNBr fragment	P-V-S-V-R-Y-D-S-P-E-F-S-G-F-S-G-S-V-Q-F-V-P-I-Q-N

FIG. 4. Comparison of the N-terminal regions of meningococcal class 1 and class 3 OMPs (strain H44/76), some gonococcal proteins I, and one meningococcal class 2 OMP (3, 4, 7).

bears homology with the recently published (4, 7) gonococcal protein I sequences beyond this point. In the meningococcal class 1 OMP, some polymorphism was observed at position 9; arginine was detected at about 30% compared to lysine.

The amino-terminal sequences of both class 1 and 3 OMPs of strain H44/76 also have high resemblance to gonococcal protein I sequences. It is clear that class 3 OMP has the same amino-terminal sequence (up to 10 residues so far) as gonococcal protein I from strain 120176-2 and that the class 1 OMP sequence is more like the class 2 OMP sequence of the meningococcus strain BNCV. The sequence data shown in Fig. 4 indicate that the N-terminal portions are conserved among the meningococcal class 1 and class 2/3 OMPs as well as in the gonococcal proteins I. This further confirms the observation that these proteins have the same function, i.e., porin, in meningococci and gonococci (8).

We thank Hanneke Versantvoort for technical assistance.

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