Highly Conserved Neisseria meningitidis Surface Protein Confers Protection against Experimental Infection

By Denis Martin, Nathalie Cadieux, Josée Hamel, and Bernard R. Brodeur

From Unité de Recherche en Vaccinologie, Centre de Recherche en Infectiologie, Centre Hospitalier Universitaire de Québec, Ste-Foy, Québec, Canada, G1V 4G2

Summary

A new surface protein, named NspA, which is distinct from the previously described Neisseria meningitidis outer membrane proteins was identified. An NspA-specific mAb, named Me-1, reacted with 99% of the meningococcal strains tested indicating that the epitope recognized by this particular mAb is widely distributed and highly conserved. Western immunoblotting experiments indicated that mAb Me-1 is directed against a protein band with an approximate molecular mass of 22,000, but also recognized a minor protein band with an approximate molecular mass of 18,000. This mAb exhibited bactericidal activity against four meningococcal strains, two isolates of serogroup B, and one isolate from each serogroup A and C, and passively protected mice against an experimental infection. To further characterize the NspA protein and to evaluate the protective potential of recombinant NspA protein, the nspA gene was identified and cloned into a low copy expression vector. Nucleotide sequencing of the meningococcal insert revealed an ORF of 525 nucleotides coding for a polypeptide of 174 amino acid residues, with a predicted molecular weight of 18,404 and a isoelectric point of 9.93. Three injections of either 10 or 20 μg of the affinity-purified recombinant NspA protein efficiently protected 80% of the mice against a meningococcal deadly challenge comparatively to the 20% observed in the control groups. The fact that the NspA protein can elicit the production of bactericidal and protective antibodies emphasize its potential as a vaccine candidate.

Neisseria meningitidis causes both endemic and epidemic diseases, principally meningitis and meningococcemia (1, 2). This pathogenic bacteria primarily affects young children between 6 mo and 2 yr of age, but often infects teenagers (1). The incidence per year of meningococcal diseases during endemic periods is normally ~1–3 cases per 100,000 in developed countries, but it can be as high as 500 per 100,000 during epidemics (2, 3).

N. meningitidis is classified into 12 serogroups based on the immunological characteristics of the capsular polysaccharides found at their surface. Within serogroups, different serotypes, subtypes, and immunotypes can be identified based on the antigenic specificity of the major outer membrane (OM)1 proteins and LPS (4). Approximately 90% of all meningococcal diseases worldwide are caused by isolates of serogroups A, B, and C (5). Vaccines based on the capsular polysaccharides of serogroups A, C, W-135, and Y were developed and proved efficient to control outbreaks and epidemics of meningococcal diseases (6). However, these vaccines are poorly immunogenic in very young children. Moreover, they do not induce immunological memory and the duration of the protection they provide is relatively short (5, 7–11). Recently, it was demonstrated that conjugation of capsular polysaccharides of serogroups A and C to carrier proteins resulted in a better immunogenicity and a longer persistence of specific antibodies against isolates of these serogroups (12–16). Attempts to develop an efficient vaccine against serogroup B isolates, which are responsible for 50–70% of the meningococcal disease in the developed countries were unsuccessful because the group B capsular polysaccharide is not a good immunogen in human, inducing only a poor IgM response of low specificity which is not protective (17–19). Furthermore, the presence of closely similar, cross-reactive structures in the glycoproteins of neonatal human brain tissue might discourage attempts to improve the immunogenicity of serogroup B polysaccharide (10).

To develop a vaccine effective against meningococci of serogroup B several non-capsular surface structures are under investigation (6, 10). Importantly, the presence of bactericidal antibodies against N. meningitidis have been strongly correlated with human immunity and protection (20–22). For that reason, it is believed that non-capsular surface antigens shown to stimulate bactericidal antibodies should be considered as the prime vaccines candidates (6). Early stud-

1Abbreviations used in this paper: pl, isoelectric point; OM, major outer membrane; Opa, opacity protein; PVDF, polyvinylidene difluoride.
ies using sera of immunized volunteers and convalescent patients indicated that certain meningococcal surface proteins such as the ones responsible for serotype specificity and LPS could induce bactericidal antibodies and be involved in protection (23, 24). mAbs were then used to clearly establish the protective potential of certain meningococcal major surface proteins such as the PorA (class 1), PorB (class 2/3), and Opc (class 5C) (25–28).

Different vaccines based on OM proteins were recently evaluated in clinical trials and efficiency between 50 and 80% were recorded (6, 10). These first generation OM proteins vaccines often induced protection against a limited number of strains. Thus, these vaccines could be used during meningococcal epidemics when the antigenic variation of the meningococci causing diseases is relatively low. The specificity of the bactericidal antibodies induced by these vaccines was determined to be directed mainly against PorA and Opc proteins (29, 30). However, the PorA-specific bactericidal antibodies were found to be directed against epitopes located in surface-exposed highly variable regions (31). Moreover, the Opc protein was shown to be produced by only 60% of strains of different serogroups (32), and by ~20% of serogroup B isolates (33). To improve the protection conferred by the PorA protein, strategies such as multivalent PorA vaccines or the incorporation of additional epitopes on PorA protein are presently under study (34–36). Proteins induced by iron limitation such as FrpB and Tbp-2 are also likely vaccine candidates, but they also show type specificity with respect to the induction of bactericidal antibodies (37–40). Antibody specific proteins such as the Lip (or H.8) (41, 42) and the R mp (or class 4) (43) proteins were identified in the meningococcal OM, but antibodies directed against these proteins were found to be nonbactericidal. Moreover, high concentrations of antibodies to the R mp protein were also reported to block the bactericidal activity of antibodies directed against PorA protein and could prevent the efficient killing of meningococcal cells (43).

In the present report, we described a new highly conserved protein, called Nspa for N. meningitidis surface protein A, which was shown to be present in the OM of all N. meningitidis strains tested. A mAb, named M e 1 , which is directed against the Nspa protein was found to be bactericidal and to passively protect BALB/c mice against experimental infections. To further characterize this protein and to clearly establish its protective potential, the Nspa gene was identified, sequenced and cloned into a low copy expression vector to obtain large quantities of the recombinant protein. In addition, we present data that demonstrate that the injection of purified recombinant Nspa protein efficiently protect BALB/c mice against a bacterial challenge with a lethal dose of a meningococcal strain of serogroup B.

Materials and Methods

Bacterial Strains and Plasmids. A collection of 250 N. meningitidis strains was used in this study. The panel of strains included 22 isolates of serogroup A, 44 isolates of serogroup B, 56 isolates of serogroup C, 1 isolate of serogroup 29-E, 7 isolates of serogroup W-135, 1 isolate of serogroup X, 3 isolates of serogroup Y, 2 isolates of serogroup Z, and 114 isolates that were not serogrouped. The following N. meningitidis species were also tested: 43 N. gonorrhoeae, 4 N. cinerea, 7 N. lactamica, 1 N. flavca, 1 N. flavescens, 3 N. mucosa, 4 N. perflavca/sica, 4 N. perflava, 1 N. sica, 1 N. subflava and 5 M. oxyridaxlal catarrhalis. The N. meningitidis strains were grown overnight on chocolate agar plates at 37°C in an atmosphere containing 5% CO2. Cultures were stored at −70°C in heart infusion broth (1.5°C Laboratories, Detroit, MI) with 20% (vol/vol) glycerol (Sigma Chemical Co., St. Louis, MO). These isolates were obtained from the Caribbean Epidemiology Centre (Port of Spain, Trinidad); Children's Hospital of Eastern Ontario (Ottawa, Canada); Department of Saskatchewan Health (Regina, Canada); Laboratoire de Santé Publique du Québec (Montreal, Canada); Max-Planck Institut fur Molekulare Genetik (Berlin, Germany); Montreal Children Hospital (Montreal, Canada); Victoria General Hospital (Halifax, Canada); Laboratory Centre for Disease Control (Ottawa, Ontario), and our own strains collection.

The following control bacterial species were also used: A laligenes faecalis (ATCC 8750); American Type Culture Collection, Rockville, MD); Bordetella pertussis 9340, C lnbacter freundii (ATCC 2080); E. coli (ATCC 35474), D ectorbacterior aggeneses (ATCC 13048), Flavobacterium otorum (LSPO 2135), H. influenzae, M. pneumoniae, K. pneumoniae (ATCC 130853), P. pseudotabigen (ATCC 29532), Proteus vulgaris (ATCC 13315), S. pneumoniae (ATCC 9027), Salmonella typhimurium (ATCC 14028), Serratia marcescens (ATCC 8100), Shigella flexneri (ATCC 12022), Shigella sonnei (ATCC 9290) and Streptococcus pneumoniae W12.

E. coli strain JM 109 (endA1 recA1 gyrA96 thi-1 hsdR17 [rK- mK-]) relA1 supE44 Δ(lac-proAB) [F' traD36 proAB lacZΔM15] (44), LE392 (F− hsdR154 [rK- mK-] supE44 supF58 lacY1 Δ[lacZΔM15] tyrB galK2 galT22 metB1 trpR55 Δ1) (45), and BL21(DE3) (F−ompT hsdS(rB− mB−) galΔlacZD36) (46) were grown on LB agar or broth (GIBCO BRL, Gaithersburg, MD) at 37°C. Where appropriate, E. coli strains or transformants were grown on media containing 25 μg of ampicillin (Sigma) per ml. The low copy plasmid pK S30, which carries a gene for resistance to ampicillin, a multiple cloning region flanked by the T7 and T3 RNA promoters, the lacZα gene, and the bacteriophage f1 origin of replication for production of single stranded DNA was described in details elsewhere (47).

Antigen Preparation. Lithium chloride extractions of OM preparations from one N. gonorrhoeae strain A1 and nine meningococcal strains two of serogroup A (604B and 24063), one of serogroup B (608B [B:2a:P1.2.1,3]), two of serogroup C (2241C and 59C), one of serogroup 29-E, one of serogroup W-135, one of serogroup Y (SLATY), and one of serogroup Z (SLATZ) were performed as described by Brodeur et al. (28). Protein concentrations were determined by the Lowry method adapted to membrane fractions (48).

Generation of mAb M e 1 . Mice were injected intraperitoneally with 20 μg of OM preparation extracted from the meningococcal strain 604A, mixed with Freund's complete adjuvant (GIBCO BRL), and then 3 wk later were injected with 20 μg of OM preparation extracted from the meningococcal strain 2241C mixed with Freund's incomplete adjuvant. 3 d before the fusion procedure, the selected mouse received a final intravenous injection of 10 μg of OM preparation extracted from the meningococcal strain 2241C. The fusion protocol used to produce the hybridoma cell lines was described previously by Hamel et al. (49). Hybrid clone supernatants were tested for specific antibody produc-
tion by ELISA, as previously described (49), using meningococcal OM preparations as coating antigens (7.5 μg of protein per ml). Specific hybrids were cloned by sequential limiting dilutions, expanded, and frozen in liquid nitrogen. The class, subclass and light-chain type of the mAb were determined by ELISA with commercially available reagents (Southern Biotechnology Associates Inc., Birmingham, AL). A dot enzyme immunoblot assay was used for the rapid screening of the mAb against the bacterial strains as described elsewhere (50).

**SDS-PAGE, Western Immunoblotting, Plaque, and Colony Blot Assays.** Meningococcal OM preparations were resolved by electrophoresis by using the discontinuous buffer system of Laemmli (51) with 14% (wt/vol) gels, and Western blot analyses were performed as described previously with the following modifications (52). The proteins were transferred from the gels to the nitrocellulose membranes using a semi-dry apparatus (Bio-Rad Labs., Hercules, CA). A current of 60 mA per gel was applied for 20 min in the electroblot buffer consisting of 25 mM Tris-HCl, 192 mM glycine, and 20% (vol/vol) methanol, pH 8.3. The presence of LOS in the different antigen preparations was visualized by silver staining as described by Tsa and Frasch (53). Recombinant plaques were blotted onto nitrocellulose membranes using the following protocol. The plates were incubated 15 min at −20°C to harden the top agar, and nitrocellulose membranes were gently applied onto the surface of the plates for 30 min at 4°C to absorb the proteins produced by the recombinant viral clones. The membranes were then washed in PBS Tween 0.02% (vol/vol). The plaque blots were sequentially incubated with tissue culture supernatant of mAb M e-1, peroxidase-labeled goat anti–mouse immunoglobulin (Jackson ImmunoResearch Laboratories Inc., West Grove, PA), and the o-dianisidine (Sigma) peroxidase substrate as described previously (52). Colony blots of E. coli transformants were performed as described by (45).

**Cloning the Gene for the NspA Protein.** A XGENE-11 genomic DNA library from N. meningitidis strain 608B was constructed according to the manufacturer’s recommendations (Promega, Madison, WI). The recombinant phages were used to infect E. coli strain LE392 which was then plated onto LB agar. The plaques were screened with mAb M e-1 and the reactive clones were plaque purified twice. The plaque DNA from one positive clone was extracted and purified from 10 ml of phage lysate using the Double Stranded Nested Deletion Kit (Bio-Rad). To further reduce the size of the insert, the plaque purified twice. The phage DNA from one positive clone was extracted and purified from 10 ml of phage lysate using the LambdaSorb Phage Adsorbent following the manufacturer’s recommendations (Promega). The DNA was then digested with ClaI, and the fragments were purified by phenol extraction from low melting agarose gels, and ligated into the SacI-digested plasmid pWKS30 and transformed into E. coli strain JM109 by electroporation according to the manufacturer’s recommendations (Bio-Rad). To further reduce the size of the insert, the SacI fragment was digested with ClaI, and the fragments were purified by agarose gel electrophoresis and ligated into the ClaI-digested plasmid pWK S30 and transformed into E. coli strain JM109 by electroporation according to the manufacturer’s recommendations (Bio-Rad). To further reduce the size of the insert, the SacI fragment was digested with ClaI, and the fragments were purified by agarose gel electrophoresis and ligated into the ClaI-digested plasmid pWK S30 and the recombinant plasmid was used to transform E. coli strain JM109 by electroporation. The transformants were screened with mAb M e-1, and two plasmids recovered from two reactive clones were designated pN2202 and pN2203.

**DNA Sequencing.** The Double Stranded Nested Deletion Kit from Pharmacia Biotech Inc. (Piscataway, NJ) was used according to the manufacturer’s instructions to generate a series of nested deletions from the recombinant plasmids pN2202 and pN2203. The resulting truncated inserts were sequenced from the M 13 forward primer with the Taq Dye Deoxy terminator Cycle Sequencing Kit using an Applied Biosystems Inc. (Foster City, CA) automated sequencer model 373A according to the manufacturer’s recommendations. Both strands of the insert were sequenced. Alignment of the sequences was accomplished by using Geneworks Software (IntelliGenetics, Inc., Mountain View, CA). Sequence homology searches in GenBank and Swissprot databases were accomplished using the Geneworks, BLAST, BL2ZT, and FASTA software.

**NH2-terminal Amino Acid Sequence Analysis of the Native Meningococcal NspA Protein.** The recombinant NspA protein was purified from the culture supernatant by affinity chromatography. To generate the affinity chromatography media, mAb M e-1 was first purified from ascitic fluid using protein A-Sepharose CL-4B (Pharmacia) and was then immobilized on CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer’s instructions. To obtain the NspA recombinant protein, the plasmid pN2202 was used to transform E. coli strain BL21(DE3) by electroporation. An overnight culture of the transformed E. coli strain BL21(DE3) was inoculated in LB broth containing 25 μg/ml of ampicillin and was incubated 4–5 h at 37°C with agitation. No induction step was used since the expression of the NspA protein was under the control of the gene’s own promoter. The bacterial cells were removed from the culture media by centrifugation at 12,000 g for 30 min at 4°C. The supernatant was filtered onto a 0.22-μm membrane and concentrated using an ultrafiltration apparatus (Amicon Inc., Beverly, MA) and a membrane with a molecular cut off of 10,000. To solubilize the membrane vesicles, Empigen BB (Carbiochem N ovabiochem, La Jolla, CA) was added to the concentrated culture supernatant to a final concentration of 1% (vol/vol), incubated at room temperature for one h, and then dialyzed overnight against 10 mM Tris-HCl buffer, pH 7.3, containing 0.05% Empigen (vol/vol). The antigen preparation was added to the affinity gel and incubated overnight at 4°C with constant agitation. The gel was poured into a chromatography column and washed extensively with 10 mM Tris-HCl buffer, pH 7.3, containing 0.05% Empigen (vol/vol). The NspA protein was eluted from the column with 1 M LiCl in Tris-HCl buffer, pH 7.3. The fractions containing the NspA protein were pooled and dialyzed extensively against 10 mM Tris-HCl buffer, pH 7.3, containing 0.05% Empigen (vol/vol).

**Bactericidal Assay.** The bactericidal activity of purified mAb M e-1 was tested in vitro as described previously (55) with the following modifications. 10 μl of a meningococcal suspension adjusted to 3 × 108 CFU/ml was dispensed into the wells of sterile flat-bottom 96-well plates (GIBCO BRL) containing 70 μl of PBS with 0.15 mM CaCl2, 0.5 mM MgCl2, and 0.1% BSA, 10 μl of guinea pig serum as a source of complement, and 10 μl of different dilution of protein A purified mAb M e-1, or heat-inactivated mouse serum. Duplicate antigen/antibody mixtures were incubated either in the absence of complement or with heat-inactivated guinea pig serum. After incubation for 1 h at 37°C, 10 μl of each mixture were plated onto chocolate agar plates. The plates were incubated overnight at 37°C, after which time the bacterial colonies were counted.

**Protection Experiments.** The mouse model of infection used for the passive and active protection experiments was described.
previously (28). In brief, the N. meningitidis strain 608B (B: 2a: P1.2:L3) was passaged twice in mice before the bacterial challenge. For inoculation of mice, meningococci were removed from the chocolate agar plates after ~20 h of incubation and suspended in PBS. The bacterial suspension was adjusted to an optical density of 0.25 (λ = 490 nm) and diluted to obtain 1,000 CFU per ml in brain heart infusion broth (Difco) containing 4% mucin (from porcine stomach type II; Sigma) and 1.5% hemoglobin (Oxoid Ltd., Nepean, Canada). All inocula were verified by colony counts. The number of surviving mice were recorded 24 and 72 h after the bacterial challenge. The mice that survived a bacterial challenge were always monitored for an additional 2 wk to see if there was any sign of infection. To evaluate the protective potential of mAb M e-1, 600 μl of ascitic fluid per mouse was injected intraperitoneally 18 h before the challenge. The mice in the control groups received the same volume of ascitic fluid containing an unrelated mAb or PBS. For the active protection experiments, groups of mice were injected three times at 3-wk intervals with 10 or 20 μg of affinity-purified NspA recombinant protein and 25 μg of Q uilA (CedarLane Laboratories, Hornby, Ontario, Canada) as the adjuvant. Control mice were injected with either 20 μg of BSA (Sigma), concentrated E. coli BL21(DE3) supernatant, or PBS. 2 wk after the third injection the mice were used for the protection experiments. Blood samples were collected via the periorbital sinus before each immunization and 10 d after the third injection for mice receiving 20 μg of purified NspA protein or E. coli concentrated supernatant. The specific titer of each of these sera was determined by ELISA against meningococcal OM preparation extracted from the meningococcal strain 608B. The serum dilution for which an absorbance reading of 0.1 (λ = 410/630 nm) was recorded after background subtraction was considered to be the titer of this serum. The bactericidal activity of these sera was evaluated as described previously and the serum dilution giving 50% killing of meningococci was considered to be the bactericidal titer.

Results

Identification of the Meningococcal NspA Protein. Mice were immunized with different combinations of meningococcal OM preparations in order to obtain hybridoma clones secreting mAbs specific for highly conserved surface antigens. mAb M e-1 was derived from one of these fusion experiments. The mouse that was used for that particular fusion was successively immunized with OM preparations from one serogroup A strain (604A) and one serogroup C strain (2241C). mAb M e-1 was selected because it reacted with all nine meningococcal OM preparations tested, but did not recognize the OM preparation extracted from N. gonorrhoeae strain A1. The hybridoma cell line secreting mAb M e-1 was cloned twice by limiting dilution. The class, subclass, and light chain specificity of this mAb were determined to be IgG2ax. A dot enzyme assay using whole cell preparations as the source of antigen was used to evaluate the specificity and the antigen conservation of the epitope recognized by mAb M e-1. mAb M e-1 reacted with 248 out of the 250 meningococcal strains tested (99.2%). The panel of strains used for the dot assay is described in detail in Materials and Methods. Only one non-typable meningococcal strain and one serogroup B strain were not recognized by the mAb. This result clearly indicates that the epitope recognized by this particular mAb is widely distributed among meningococcal strains. It also suggests that the particular epitope recognized by mAb M e-1 is not restricted to serologically related isolates since the panel of meningococcal strains were chosen to represent the major disease causing groups of strains. Moreover, two other NspA-specific mAbs, called M e-2 and M e-7, which were generated from other fusion experiments, reacted with the two meningococcal strains that were not recognized by mAb M e-1 (56). This latter result indicates that the epitopes recognized by these NspA-specific mAbs are present in the OM of all meningococcal strains tested so far. The reactivity of the mAb M e-1 was also evaluated with other N. menin-gococcal strains. Out of the 74 non-meningococcal strains tested, mAb M e-1 reacted only with one N. lactamica strain, but not with any other N. meningitidis strains tested. This mAb did not recognize any other gram-negative and gram-positive bacterial strains tested.

Western immunoblotting experiments indicated that mAb M e-1 reacted with a protein band with apparent molecular mass of 22,000 (Fig. 1 B). This mAb also reacted with a minor protein band with apparent molecular mass of 18,000. To observe the minor band, the total protein concentration of 5 μg that is normally applied to each well had to be increased to 7.5 or 10 μg. Analysis of SDS-PAGE and Western immunoblots indicated that the variation normally observed in the OM protein migration profiles, the molecular mass of the 22,000 protein band is constant among meningococcal isolates. However, the amount of 22-kD protein band present in the different OM preparations varied from one meningococcal strain to another.

Cloning of the nspA Gene. A meningococcal chromosomal library was constructed from N. meningitidis strain 608B in LambdaGEM-11. The genomic DNA was partially digested with Sau3AI and fragments ranging between 9 and 23 kb were purified and ligated to the BamHI sites of the LambdaGEM-11 bacteriophage arms. 19 positive plaques were identified after the immunoscreening of the library.
with mAb Me-1. After amplification and DNA purification, one viral clone which had a 13-kb insert was selected for the subcloning experiments. After digestion of this clone with SacI, two fragments of 5 and 8 kb were obtained, purified, and ligated into the SacI restriction site of the plasmid pWKS30.

*E. coli* strain JM109 transformed with the recombinant plasmids were screened with mAb Me-1. Positive colonies were observed only when the bacteria were transformed with the plasmid carrying the 8-kb insert. Western blot analysis of the positive clones showed that the protein expressed by *E. coli* strain JM109 was complete and migrated on SDS-PAGE gel like the *N. meningitidis* NspA protein.

A purified 8-kb fragment obtained from a positive clone was digested with ClaI to further reduce the size of the insert. The resulting 2.75-kb fragment was then ligated into the ClaI site of the pWKS30 plasmid. Western blot analysis of the resulting clones clearly indicated once again that the protein expressed by *E. coli* strain JM109 was complete and migrated on SDS-PAGE gel like the native *N. meningitidis* NspA protein (Fig. 2, lane 2). The arrow indicates the location of the 22-kD NspA protein band. On the left, mol wt standard proteins are Phosphorylase b (97,400), BSA (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400).

Sequence Analysis of nspA.

The two plasmids pN2202 and pN2203 were selected to proceed with the sequencing of the N. meningitidis strain 608B NspA protein. The open reading frame of 525 bp extends from the start codon at base 143 to the stop codon at base 667. A 19-amino acid leader peptide is underlined, and a putative ribosome binding site is boxed. These sequence data are available from GenBank under accession number U52066.

**Figure 3.** Nucleotide and amino acid sequences of the gene encoding the *N. meningitidis* strain 608B NspA protein. The open reading frame of 525 bp extends from the start codon at base 143 to the stop codon at base 667. A 19-amino acid leader peptide is underlined, and a putative ribosome binding site is boxed. These sequence data are available from GenBank under accession number U52066.
other known membrane proteins (57). The low amount of 18-kD protein band present in the meningococcal OM preparation after separation on SDS-PAGE prevented the same analysis. For that reason, affinity-purified recombinant protein was used to overload a gel in order to obtain enough of the minor protein band to resolve its N-terminal amino acid sequence. The first five amino acid residues of the minor 18-kD band were determined to be E-G-A- S-G-F, which corresponded exactly to the first amino acid residues of the mature meningococcal protein without its leader peptide. This result and the observed reactivity of mAb Me-1 with both protein bands suggest that the 22- and the 18-kD protein bands are related and possibly are the same protein.

No significant homology was found between the deduced amino acid sequence of the NspA protein and the sequences compiled in GenBank and Swissprot databases indicating that this protein has never been described previously. These searches indicated the existence of weak homology of the NspA protein with the Neisseria opacity (Opa) family of protein. Pairwise comparison of the different Opa protein sequences with the NspA sequence revealed identities between 20 and 28%. Closer analysis indicated that the observed homology with the Opa proteins seems to be clustered in two particular regions on the NspA protein. The first region that showed 62.5% homology with some of the Opa proteins is composed of 24 amino acid residues located between position 126 and 149. The second region which showed 60% homology with the carboxy-terminal regions of Opa proteins, is made up of the last 10 amino acid residues at the carboxy-terminal of the NspA protein.

Production and Purification of the Recombinant NspA Protein. The purification protocol was based on the observation that the recombinant NspA protein produced by E. coli BL21(DE3) strain carrying the plasmid pNP2202 can be found in large amounts in the OM, but can also be obtained from the culture supernatant in which it is one of the most abundant proteins (Fig. 2A). Preliminary experiments indicated that an induction step using isopropyl-β-d-thiogalactopyranoside (IPTG) was not necessary since it did not significantly increase the level of NspA protein which was constitutively produced by E. coli BL21(DE3) strain transformed with plasmid pNP2202. This result indicated that this neisserial promoter can efficiently control the expression of the nspA gene in E. coli. Therefore, the culture supernatant was the material used to purify the recombinant NspA protein using affinity chromatography. Silver-stained gel indicated that the amount of E. coli LPS present after affinity chromatography is greatly reduced compared to the amount initially present in the concentrated supernatant (Fig. 2A). The recombinant NspA protein was still recognized by mAb Me-1 after solubilization and purification (Fig. 2C). The purity of the NspA protein used to immunize mice was estimated by SDS-PAGE to be >90%.

Determination of the Bactericidal Activity and the Protective Potential of mAb Me-1. To demonstrate the bactericidal activity of mAb Me-1 in an in vitro assay was performed using guinea pig serum and protein A-purified mAb. The bactericidal activity of mAb Me-1 against the meningococcal strain 608B is presented in Fig. 5. 7 μg/ml of purified mAb Me-1 were required to kill 50% of the meningococcal cells. Higher concentrations of purified mAb resulted in a sharp decrease in the recorded CFU ≤100%. Heat-inactivation for 30 min at 56°C of the guinea pig serum completely abolished the bactericidal activity of mAb Me-1. 50% of killing were recorded for three other meningococcal strains, one of each serogroup A (strain Z4063), B (B: 15:P1.7) strain 164), and C (strain 3), when 20 μg/ml of purified M e-1 was used in the bactericidal assay.

The ability of mAb Me-1 to passively protect mice against a lethal infection with the meningococcal strain 608B was evaluated and the combined results from three separate experiments are presented in Table 1. Intraperitoneal injection of 600 μl of ascitic fluid containing mAb Me-1 18 h before the bacterial challenge with 1,000 CFU increased the survival rate of mice ≤76% comparatively to 30% observed in the control groups receiving ascitic fluid containing either an unrelated mAb or PBS.

Immunization of BALB/c mice with Affinity-purified Recombinant NspA Protein and Protection Experiments. Affinity-puri-
fied recombinant meningococcal NspA protein was used to immunize BALB/c mice in order to determine its ability to induce a protective immune response against challenge with a lethal dose of N. meningitidis 608B strain (Table 2). 80% of the mice immunized with three injections of either 10 or 20 μg of purified recombinant meningococcal NspA protein survived the bacterial challenge comparatively to 0 to 20% in the control groups. Survivors at 72 h did not succumb during an additional two weeks of observation. The mice in the control group injected with concentrated E. coli culture supernatant were not protected against the bacterial challenge indicating that the components present in the culture media and other E. coli antigens that might be present in small amounts after purification do not contribute to the observed protection against N. meningitidis.

Serum samples were obtained before immunization and after the third injection but before the bacterial challenge for mice immunized with 20 μg of affinity-purified protein or E. coli concentrated supernatant. The titer of these sera were determined by ELISA using meningococcal OM preparation as coating antigen. The reciprocal serum titers varied from 4,800 to 51,200 for the mice injected with 20 μg of protein, but were below 200 for the mice in the control group. Western immunoblotting experiments confirmed that the antibodies present in the sera obtained from the immunized mice recognized the recombinant protein, but also reacted with the native meningococcal NspA protein present in the OM preparation (data not shown). The reciprocal bactericidal titers of these sera varied from 4,800 to 51,200 for the mice injected with 20 μg of purified NspA protein 9/10 8/10 80

Table 1. Passive Protection of BALB/c Mice Conferred by mAb Me-1 Against Infection with N. meningitidis Strain 608B

| Groups                  | % of survival (range)‡ |
|-------------------------|-------------------------|
| M e-1-injected mice      | 24 h 72 h                |
| Control mice             | 10/30 9/30 30           |
|                          | (60–100) (0–40)         |

*The mice were challenged with 1 ml of a suspension containing 1,000 CFU of N. meningitidis strain 608B, 4% mucin (Sigma), and 1.6% hemoglobin (Oxoid).
† Combined results of three separate protection experiments. The range indicates the lowest and highest survival rates obtained.
‡ 600 μl of ascitic fluid containing mAb Me-1 were injected intraperitoneally 18 h before the bacterial challenge.
§ The mice in the control group received 600 μl of ascitic fluid containing unrelated mAb or phosphate buffered saline.

bactericidal antibodies, and correlated well with the ELISA titers. No bactericidal activity was recorded for the sera obtained from mice in the control group. Analysis of the sera obtained from the two mice who died following the bacterial challenge revealed that they had the lowest ELISA titers (4,800) as well as no bactericidal activity.

### Discussion

In addition to the well known major OM proteins, it is believed that other antigenically stable surface proteins could play an important role in the protection against N. meningitidis and could become for that reason interesting vaccine candidates. Already, it was demonstrated that minor meningococcal antigens such as iron-regulated proteins could induce bactericidal antibodies that protected mice against experimental infection (37, 38). We have identified a minor antigen which is present in the OM of all meningococcal strains tested so far. To our knowledge this protein, which was called NspA for N. meningitidis protein A, was not described previously. Interestingly, comparison of the deduced amino acid sequence obtained after sequencing the nspA gene and the protein sequences compiled in the available databases revealed that two regions located between amino acid residues 126 and 149 and the last 10 residues at the carboxyl end showed ~60% homology with members of the Neisseria opacity protein (Opa) family of proteins. These Opa proteins appear to be involved in cells adherence and invasion of epithelial or endothelial human cells (58). The observed similarity is clustered in two regions that were described to be highly conserved among Opa proteins (59, 60). The first conserved region is located between amino acid residues 126 and 149 and the last 10 residues at the carboxyl end showed ~60% homology with members of the Neisseria opacity protein (Opa) family of proteins. These Opa proteins appear to be involved in cells adherence and invasion of epithelial or endothelial human cells (58). The observed similarity is clustered in two regions that were described to be highly conserved among Opa proteins (59, 60). The first conserved region is located between amino acid residues 126 and 149 and the last 10 residues at the carboxyl end showed ~60% homology with members of the Neisseria opacity protein (Opa) family of proteins. These Opa proteins appear to be involved in cells adherence and invasion of epithelial or endothelial human cells (58). The observed similarity is clustered in two regions that were described to be highly conserved among Opa proteins (59, 60). The first conserved region is located
on Opa proteins between two highly variable regions, named HV1 and HV2, which display considerable heterogeneity in sequence and in length, while the second region is found at the carboxyl end. These results suggest that the NspA protein could somehow be a smaller homologue of the Opa proteins and for that reason could be implicated in the meningococcal pathogenesis. However, beside the observed homology in the conserved regions, the NspA protein does not share two of the well-known characteristics of the Opa proteins such as the antigenic variability and the translational control of their expression during phase variation by multiple repeats of the nucleotide sequence (CTCTT) (59, 60). This homology can also be explained by the fact that the two regions of the NspA protein could be included in functional domains that are common to several OM proteins. Indeed, these two regions are hydrophobic as determined by a Kyte-Doolittle analysis (Fig. 4) and could be part of transmembrane domains which would normally be antigenically conserved and embedded in the meningococcal OM. More information about the physico-chemical properties and the mechanism which control the expression of the proteins is needed to clearly associate the NspA protein with the Opa proteins or to any other known meningococcal surface protein.

Even if the biological function of the NspA protein is not known at the moment, it is possible to determine using mAb M-e-1 whether this protein can induce an immune response that can protect against meningococcal infection. MAb M-e-1 was shown to be bactericidal against four meningococcal strains, one serogroup A, two serogroup B, and one serogroup C, indicating that the observed bactericidal activity is not restricted to one particular strain. It has been well documented that serum bactericidal activity is the major defense mechanism against N. meningitidis and that protection against invasion by the bacteria correlates with the presence in human sera of anti-meningococcal antibodies (20, 21). Conclusive evidence for the importance of bactericidal antibodies came from observations that individuals with intact opsonophagocytic capacity (61), but with deficiencies in one of the terminal complement components, have enhanced risk of contracting meningococcal infections (62). To evaluate whether the observed bactericidal activity of mAb M-e-1 is not limited to a particular group of strains, we are presently evaluating its activity against a larger panel of serologically distinct meningococcal strains in the presence of human complement. Additional information about the localization of the NspA protein at the surface of intact meningococcal cells can also be deduced from the bactericidal activity of mAb M-e-1. Already, analysis of the migration profiles of meningococcal OM preparations have shown that the NspA protein is present in these preparations (Fig. 1). The bactericidal assay results indicate that the epitope recognized by mAb M-e-1 is exposed at the surface of intact meningococcal cells where it is accessible to the antibodies. We are presently using a radioimmunobinding assay (56) and immunoelectron microscopy to further study the exposure of the NspA protein at the surface of intact meningococcal cells and its accessibility to specific antibodies. It is important to note that unlike the common occurrence for OM proteins to have their highly conserved regions embedded in the membrane while their variable regions are surface exposed (63, 64), the epitope on the NspA recognized by mAb M-e-1 is accessible at the surface of intact bacteria. Furthermore, mAbs can be used with different animal models of infection to demonstrate the protective potential of a particular bacterial antigen. Using this approach, mAbs directed against PorA (25–27), PorB (25, 27, 28), Opc (27, 65), and LPS (27, 66) were shown to passively protect against meningococcal experimental infection. The injection of the bactericidal mAb M-e-1 considerably improved the survival rate of the mice against a deadly infection with the homologous meningococcal strain 608B (Table 1). The combined results obtained using the bactericidal assay and the passive protection clearly demonstrated that antibodies that are specific for the NspA protein can efficiently protect against an experimental meningococcal infection. A new set of experiments will have to be conducted in order to determine the extent of protection conferred by mAb M-e-1 when other serologically distinct strains are used to challenge the mice.

There are several advantages in favor of recombinant proteins for vaccine development: production of large amount of protein, lack of contaminating undesirable material, and the possibility to genetically modify the protein to increase its immunogenicity or to reduce its toxicity. For all these reasons we decided to use recombinant NspA protein instead of the native protein to clearly establish the protective potential of this meningococcal minor surface antigen. It is important to ascertain that the configuration of the recombinant protein is close enough to the native protein to generate functionally active antibodies. Indeed, Idänpää-Heikkilä et al. (67) reported the production of recombinant PorA protein in Bacillus subtilis as inclusion bodies. They observed that the native-like epitopes necessary to generate bactericidal and protective antibodies were present only when the recombinant PorA protein was allowed to refold in the presence of LPS or when it was incorporated into liposome. In our case, analysis of migration profiles by SDS-PAGE of different antigenic preparations extracted from E. coli strain BL21(DE3) transformed with the plasmid pN2202 indicated that the recombinant NspA protein is found in the OM as well as secreted in the culture supernatant (Fig. 3). Since there was no extensive solubilization and extraction steps necessary, we decided to purify the recombinant NspA protein from the culture supernatant by affinity chromatography. We hypothesized that the recombinant NspA protein, which was secreted in the culture supernatant through the bacterial OM, would be correctly folded and would generate functionally active antibodies. Data obtained from the protection experiments and the subsequent analysis of the immune sera confirmed that the recombinant protein shared enough immunological characteristics with the native protein to induce such antibodies. Indeed, 80% of the mice immunized with the
recombinant NspA protein survived a deadly meningococcal challenge (Table 2). This result clearly linked the observed protection to the development of a specific immune response. Analysis of in vitro bactericidal results also indicated that cross-reactive bactericidal antibodies were present in the sera obtained from all the mice who survived the bacterial challenge.

In this report, we have presented results demonstrating that the recombinant NspA protein can induce an immune response that can protect against a lethal meningococcal infection. We also presented data indicating that this protein is present in the OM of all meningococcal strains where it is accessible to the antibodies at the surface of living cells. For all these reasons we believe that this protein possesses all the important characteristics to be considered as a potential vaccine candidate. We are presently constructing meningococcal mutant strains in order to study the function of the NspA protein and to determine its possible role in the meningococcal pathogenesis.

Reference

1. Peltola, H. 1983. Meningococcal disease: still with us. Rev Infect. Dis. 5:71–91.
2. Schwartz, B., P.S. Moore, and C.V. Broome. 1989. Global epidemiology of meningococcal disease. Clin. Microbiol. Rev. 2(Suppl.):S118–S124.
3. Witting, H.C., and B.M. Greenwood. 1976. Meningococcal meningitis in the northern savanna of Africa. Trop. Dis. Oct. 6: 99–104.
4. Frasch, C.E., W.D. Zollinger, and J.T. Poolman. 1988. Serotype antigens of Neisseria meningitidis and a proposal scheme for designation of serotypes. Rev. Infect. Dis. 1:504–510.
5. Frasch, C.E. 1989. Vaccines for prevention of meningococcal disease. Clin. Microbiol. Rev. 2(Suppl.):S134–S138.
6. Frasch, C.E. 1995. Meningococcal vaccines: past, present and future. In Meningococcal Disease. K. Cartwright, editor. John Wiley & Sons Ltd, New York, NY. 245–283.
7. Artenstein, M.S., R. Gold, J.G. Zimmerly, F.A. Wyle, H. Schneider, and C. Harkins. 1970. Prevention of meningococcal disease by group C polysaccharide vaccine. N. Engl. J. Med. 282:417–420.
8. Peltola, H., P.H. Mäkelä, H. Kahty, H. Jousmies, E. Herä, K. Häälström, A. Sivonen, O.-V. Rinkonen, O. Pettay, V. Karanko et al. 1977. Clinical efficacy of meningococcal group A capsular polysaccharide vaccine in children three months to five years of age. N. Engl. J. Med. 297:686–691.
9. Ringold, A.L., C.V. Broome, A.W. Rightower, G.W. Ajello, G.A. Bolan, C. Adamsbaum, E.E. Jones, C. Phillips, H. Tiendrebeogo, and A. Yada. 1985. Age specific difference in duration of clinical protection after vaccination with meningococcal polysaccharide A vaccine. Lancet. 2:114–118.
10. Poolman, J.T. 1995. Development of a meningococcal vaccine. Infect. Agents Dis. 4:13–28.
11. Lepow, M.L., I. Goldschneider, R. Gold, M. Randolph, and E.C. Gotschlich. 1977. Persistence of antibody following immunization of children with groups A and C meningococcal polysaccharide vaccines. Pediatrics. 60:673–680.
12. Costantino, P., S. Viti, A. Podda, M.A. Velmonte, L. Neonici, and R. Rappuoli. 1992. Development and phase I clinical testing of a conjugate vaccine against meningococcus A and C. Vaccine. 10:691–698.
13. Jennings, H.J., and C. Lugowski. 1981. Immunochrometry of groups A, B, and C meningococcal polysaccharide-tetanus toxoid conjugates. J. Immunol. 127:1011–1018.
14. Beuvery, E.C., F. Miedema, R. van Delft, and K. Haverkamp. 1983. Preparation and immunochromatographic characterization of meningococcal serogroup C polysaccharide-tetanus toxoid conjugates as a new generation of vaccines. Infect. Immun. 40:39–45.
15. Beuvery, E.C., A. Kaaden, V. Kanhai, and A.B. Leussink. 1983. Physiochemical and immunochromatographic characterization of meningococcal serogroup A polysaccharide-tetanus toxoid conjugates prepared by two methods. Vaccine. 1:31–36.
16. Anderson, E.L., T. Bowers, C.M. Mink, D.J. Kennedy, R.B. Belshe, H. Harakeh, L. Paas, P. Holder, and G.M. Carlone. 1994. Safety and immunogenicity of meningococcal A and C polysaccharide conjugate vaccine in adults. Infed. Immun. 62: 3391–3395.
17. Zollinger, W.D., and E. Morán. 1991. Meningococcal vaccines—present and future. Trans. Roy. Soc. Trop. Med. Hyg. 85(Suppl.):37–43.
18. Mandrell, R.E., and W.D. Zollinger. 1982. Measurement of antibodies to meningococcal serogroup B polysaccharide: Low avidity binding and equilibrium binding constants. J. Immunol. 129:2172–2177.
19. Zollinger, W.D., and R.E. Mandrell. 1983. Importance of complement source in bactericidal activity of human antibody and murine monoclonal antibody to meningococcal group B polysaccharide. Infect. Immun. 40:257–264.
20. Goldschneider, I., E.C. Gotschlich, and M.S. Artenstein. 1969. Human immunity to the meningococcus. I. The role of antibodies. J. Exp. Med. 129:1307–1326.
21. Goldschneider, I., E.C. Gotschlich, and M.S. Artenstein. 1969. Human immunity to the meningococcus. II. Development of natural immunity. J. Exp. Med. 129:1327–1378.
22. Gotschlich, E.C., I. Goldschneider, and M.S. Artenstein. 1969. Human immunity to the meningococcus. IV. Immunogenicity of serogroup A and serogroup C polysaccharides in human volunteers. J. Exp. Med. 129:1367–1384.
23. Frasch, C.E., and S.S. Chapman. 1973. Classification of Neisseria meningitidis serogroup b into distinct serotypes. III. Ap-
plication of a new bactericidal inhibition technique to distribution of serotypes among cases and carriers. J. Infect. Dis. 127:149–154.
24. Jones, D.M., and J. Eldridge. 1979. Development of antibodies to meningococcal protein and lipopolysaccharide antigens in healthy carriers. J. Med. Microbiol. 12:107–111.
25. Saukkonen, K., H. Abdillahi, J.T. Poolman, and M. Leinonen. 1987. Protective efficacy of monoclonal antibodies to class 1 and class 3 outer membrane proteins of N. meningitidis B:15:P1.16 in infant rat infection model: new prospects for vaccine development. M.icrob. Pathog. 3:261–267.
26. Saukkonen, K., M. Leinonen, H. Abdillahi, and J.T. Poolman. 1989. Comparative evaluation of potential components for group B meningococcal vaccine by passive protection in the infant rat model and in vitro bactericidal assay. V.accine. 7:325–328.
27. Frasch, C.E., C.-M. Tsai, and L.F. Mocca. 1986. Outer membrane proteins of N. meningitidis: structure and importance in meningococcal disease. Clin. Invest. Med. 9:101–107.
28. Brodeur, B.R., Y. Larose, P. Tsang, J. Hamel, F. Ashton, and A. Ryan. 1985. Protection against infection with N. meningitidis group B serotype 2b by passive immunization with serotype-specific monoclonal antibody. Infect. Immun. 50:510–516.
29. Høiby, E.A., E.A. Rosenqvist, L.O. Frøholm, G. Bjune, B. Feiring, H. Naaktgebly, and E. Rønnild. 1991. Bactericidal antibodies after vaccination with the Norwegian meningococcal serogroup B outer membrane vesicle vaccine: a brief survey. NIPH (Natl. Inst. Public Health) Ann. (O SLO ). 14:147–56.
30. Zollinger, W.D., J. Bosdjo, E. Moran, J. Garcia, C. Cruz, S. Ruiz, B. Brandt, M. Martinez, J. Arthur, P. Underwood et al. 1991. Meningococcal serogroup B vaccine production trial and follow-up studies in Chile. NIPH (Natl. Inst. Public Health) Ann. (O SLO ). 14:211–213.
31. van der Ley, P.A., J.E. Heckels, M. Virji, P. Hoogerhout, and J.T. Poolman. 1991. Topology of outer membrane proteins in pathogenic N. meningitidis spp. Infect. Immun. 59:2963–2971.
32. Olyhoek, A.J., J. Sarkari, M. Bopp, G. Morelli, and M. Achtman. 1991. Cloning and expression in E. coli of the gene for an unusual class 5 outer membrane protein from N. meningitidis. M.icrob. Pathog. 11:249–257.
33. Rosenqvist, E., E.A. Høiby, E.W. edege, B. Kusecek, and M. Achtman. 1993. The 5c protein of N. meningitidis is highly immunogenic in humans and induces bactericidal antibodies. J. Infect. Dis. 167:1065–1073.
34. van der Ley, P.A., J. van der Biezen, C.C.A.M. Peeters, and J.T. Poolman. 1993. Use of transformation to construct antigenic hybrids of the class 1 outer membrane protein in N. meningitidis. Infect. Immun. 61:4724–4733.
35. van der Ley, P.A., J. van der Biezen, and J.T. Poolman. 1995. Construction of N. meningitidis strains carrying multiple chromosomal copies of the porA gene for use in the production of a multivalent outer membrane vesicle vaccine. V.accine. 13:401–407.
36. van der Ley, P.A., and J.T. Poolman. 1992. Construction of multivalent class 1 OMP expressing meningococcal vaccine strain. Infect. Immun. 60:3156–3161.
37. Danve, B., L. Lissolo, M. Mignon, P. Dumas, P.P. Colombani, S. Colombani, A.B. Schryvers, and M.J. Quentin-Millet. 1993. Transferrin-binding proteins isolated from N. meningitidis elicit protective and bactericidal antibodies in laboratory animals. V.accine. 11:1214–1220.
38. Pettersson, A., A.J. Kulmers, M. Pelzer, E.P.M. Verhagen, R.H. Tienjema, J. Tommassen, and J.T. Poolman. 1990. M. monoclonal antibodies against the 70-kilodalton iron-regulated protein of N. meningitidis are bactericidal and strains-specific. Infect. Immun. 58:3036–3041.
39. Bhatnagar, N.B., and C.E. Frasch. 1990. Expression of N. meningitidis iron-regulated outer membrane proteins including a 70-Kilodalton transferrin receptor, and their potential for use as vaccines. Infect. Immun. 58:2875–2881.
40. AalAaldeen, D.A., and S.P. Borriello. 1996. The meningococcal transferrin-binding proteins 1 and 2 are both surface exposed and generate bactericidal antibodies capable of killing homologous and heterologous strains. V.accine. 14:49–53.
41. Cannon, J.G., W.J. Black, I. Achtman, and P.W. Stewart. 1984. Monoclonal antibody that recognizes an outer membrane antigen common to the pathogenic N. meningitidis species. Infect. Immun. 43:994–999.
42. Bhattacharjee, A.K., E.E. Moron, and W.D. Zollinger. 1990. Antibodies to meningococcal H.8 (Lip) antigen fail to show bactericidal activity. C. an. J. Med. Microbiol. 36:117–122.
43. Munkley, A., C.R. Tinsley, M. Virji, and J. E. Heckels. 1991. Blocking of bactericidal killing of N. meningitidis by antibodies directed against class 4 outer membrane proteins. M.icrobiol. Pathol. 11:447–452.
44. Yanisch-Perron, C., J. Viera, and J. Messing. 1985. Improved M.13 phage cloning vectors and host strains: nucleotide sequences of the M.13mp18 and pU C19 vectors. Gene (A ust.). 33:103–119.
45. Sambrook, J., E.F. Fritsch, and T. M. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Plainview, NY. 12.21–12.23.
46. Studier, F.W., A.H. Rosenberg, J.J. Dunn, and J.W. Dubendorff. 1989. Use of T 7 RNA polymerase to direct expression of cloned genes. In Methods Enzymol. J.N. Abelson and M.I. Simon, editors. Academic Press, Inc., San Diego, CA. 185: 60–89.
47. Wang, R.F., and S.R. Kushner. 1991. Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in E. coli. Gene (Amst.). 100:195–199.
48. Lowry, O.H., N.J. Rosenbrough, A.L. Farr, and R.J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.
49. Hamel, J., B.R. Brodeur, Y. Larose, P.S. Tsang, A. Belmaaza, and S. Montplaisir. 1987. A monoclonal antibody directed against a serotype-specific, outer membrane protein of H. influenzae type b. J. Med. M.icrobiol. 23:163–170.
50. Lussier, M., B.R. Brodeur, and S. W. Inness. 1989. Detection of N. meningitidis gonorrhoeae by dot enzyme immunoassay using monoclonal antibodies. J. Immunol. 103:373–394.
51. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. N.ature (Lond.) 227:680–685.
52. Martin, D., Y. Larose, J. Hamel, J. Lagacé, and B.R. Brodeur. 1988. H. influenzae secreting human monoclonal antibodies against H. influenzae type b. J. Immunol. 138:601–606.
53. Tsai, C.-M., and C.E. Frasch. 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. A nna. l. Biochem. 119:115–119.
54. Matsuda, P. 1987. Sequence from picolomeric quantities of proteins electrophrected on polyvinyldene difluoride membranes. J. Biol. Chem. 262:10035–10038.
55. Martin, D., M.S. Peppler, and B.R. Brodeur. 1992. Immunological characterization of the lipooligosaccharide B band of Bordetella pertussis. Infect. Immun. 60:2718–2725.
56. Martin, D., N. Cadieux, J. Hamel, and B.R. Brodeur. 1996. Identification of a highly conserved outer membrane protein of Neisseria meningitidis. Am. Soc. Microbiol. 187(Abstr.):187.
57. Michaelis, S., and J. Beckwith. 1982. Mechanism of incorporation of cell envelope proteins in Escherichia coli. Annu. Rev. Microbiol. 36:435-465.
58. Virji, M., K. Makepeace, D.J.P. Ferguson, M. Achtman, and E.R. Moxon. 1993. Meningococcal Opa and Opc proteins: their role in colonization and invasion of human epithelial and endothelial cells. Mol. Microbiol. 10:499-510.
59. Aho, E.L., J.A. Dempsey, M.M. Hobbs, D.G. Klapper, and J.G. Cannon. 1991. Characterization of the Opa (class 5) gene family of Neisseria meningitidis. Mol. Microbiol. 5:1429-1437.
60. Hobbs, M.M., A. Seiler, M. Achtman, and J.G. Cannon. 1994. Microevolution within a clonal population of pathogenic bacteria: recombination, gene duplication and horizontal genetic exchange in the Opa gene family of Neisseria meningitidis. Mol. Microbiol. 12:171-180.
61. Ross, S.C., P.J. Rosenthal, H.M. Berberic, and P. Densen. 1987. Killing of Neisseria meningitidis by human neutrophils: implications for normal and complement deficient individuals. J. Infect. Dis. 155:1266-1275.
62. Densen, P. 1989. Interaction of complement with Neisseria meningitidis and Neisseria gonorrhoeae. Clin. Microbiol. Rev. 2(Suppl.): S11-S17.
63. Martin, D., R. Munson, Jr., S. Grass, P. Chong, J. Hamel, G. Zobrist, M. Klein, and B.R. Brodeur. 1991. Mapping of B-cell epitopes on the outer membrane P2 porin protein of Haemophilus influenzae by using recombinant proteins and synthetic peptides. Infect. Immun. 59:1457-1464.
64. McGuinness, B.T., P.R. Lambden, and J.E. Heckels. 1993. Class 1 outer membrane protein of Neisseria meningitidis: epitope analysis of the antigenic diversity between strains, implications for subtype definition and molecular epidemiology. Mol. Microbiol. 7:505-514.
65. Fernandez de Cossio, M.E., M. Ohlin, M. Llano, B. Selander, S. Cruz, J. Del Valle, and C.A. Borrebaek. 1992. Human monoclonal antibodies against an epitope on the class 5c outer membrane protein common to many pathogenic strains of Neisseria meningitidis. J. Infect. Dis. 166:1322-1328.
66. Verheul, A.F., A.J. Kuipers, A.K. Braat, H.A. Dekker, C.C. Peeters, H. Snippe, and J.T. Poolman. 1994. Development, characterization, and biological properties of meningococcal immunotype L3,7,(8),9-specific monoclonal antibodies. Clin. Diagn. Lab. Immunol. 1:729-736.
67. Idänpään-Heikkilä, I., S. Muttilainen, E. Wahlström, L. Sarinen, M. Leinonen, M. Sarvas, and P.H. Mäkelä. 1995. The antibody response to a prototype liposome vaccine containing Neisseria meningitidis outer membrane protein P1 produced in Bacillus subtilis. Vaccine. 13:1501-1508.