Antigenic and Molecular Conservation of the Gonococcal NspA Protein

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A low-molecular-weight protein named NspA (neisserial surface protein A) was recently identified in the outer membrane of all Neisseria meningitidis strains tested. Antibodies directed against this protein were shown to protect mice against an experimental meningococcal infection. Hybridization experiments clearly demonstrated that the nspA gene was also present in the genomes of the 15 Neisseria gonorrhoeae strains tested. Cloning and sequencing of the nspA gene of N. gonorrhoeae B2 revealed an open reading frame of 525 nucleotides coding for a polypeptide of 174 amino acid residues, with a calculated molecular weight of 18,316 and a PI of 10.21. Comparison of the predicted amino acid sequence of the NspA polypeptides from the gonococcal strains B2 and FA1090, together with that of the meningococcal strain 608B, revealed an identity of 93%, suggesting that the NspA protein is highly conserved among pathogenic Neisseria strains. The level of identity rose to 98% when only the two gonococcal predicted NspA polypeptides were compared. To evaluate the level of antigenic conservation of the gonococcal NspA protein, monoclonal antibodies (MAbs) were generated. Four of the seven NspA-specific MAbs described in this report recognized their corresponding epitope in 100% of the 51 N. gonorrhoeae strains tested. Radioimmunobinding assays clearly indicated that the gonococcal NspA protein is exposed at the surface of intact cells.

Neisseria meningitidis and Neisseria gonorrhoeae are pathogenic Neisseria species. These species, which cause quite distinct diseases, are closely related, having more than 80% DNA genome homology and up to 98% sequence similarity for housekeeping genes (18, 40). This high degree of relatedness is reflected in their many common genetic, biochemical, and antigenic features. For example, it was shown that N. meningitidis produces proteins highly similar to the gonococcal PI (2, 12, 17, 21), PII (3, 22, 33), and PIII (6, 16) outer membrane (OM) proteins as well as the pilin protein (30, 34), the iron-repressible proteins (32), and the H.8 antigen (5, 9, 10, 16). The high levels of inter- and intraintrain antigenic variations of the OM components of N. gonorrhoeae appear to allow this organism to evade the host immune system and limit the capacity of those antigens to serve as vaccines (37). Identification of conserved antigens is of great interest, considering the high levels of heterogeneity and antigenic variations for the different gonococcal outer membrane components.

Martin et al. (28) recently reported the identification in the OM of N. meningitidis of a low-molecular-weight protein, which they named NspA (neisserial surface protein A). Using NspA-specific monoclonal antibodies (MAbs), they showed that this protein was antigenically highly conserved and accessible at the surface of intact bacterial cells of all N. meningitidis isolates tested. Two of these NspA-specific MAbs were shown to be bactericidal in vitro against several meningococcal isolates (27). Intraperitoneal injection of these bactericidal MAbs passively protected mice against a lethal meningococcal challenge. It was also demonstrated that the injection of recombinant NspA (rNspA) protein produced by Escherichia coli protected mice against experimental meningococcal infection (28).

In this study, gonococcal NspA-specific MAbs were generated to further investigate the antigenic conservation of the NspA protein. The gonococcal nspA gene was cloned and sequenced to obtain additional information about the molecular conservation of nspA genes among the two pathogenic Neisseria species.

MATERIALS AND METHODS

Bacterial strains and culture conditions. A collection of 51 clinical and laboratory strains of N. gonorrhoeae and 8 strains of N. meningitidis was used in this study. Of the N. gonorrhoeae strains, seven were isolates from patients with disseminated gonococcal infections and were provided by P. Turgeon, St-Luc Hospital, Montreal, Canada. N. gonorrhoeae FA1090 (13) and M51 (31) were kindly provided by A. Juret, Uniformed Services University of the Health Sciences, Bethesda, Md. All other strains were obtained from the culture collection of the National Reference Center for Neisseria and from the Antimicrobial and Molecular Biology Division of the Laboratory Center for Disease Control, Ottawa, Canada. The N. gonorrhoeae strains were grown overnight on chocolate agar plates (Quelab Laboratories, Montreal, Canada) at 37°C in an atmosphere containing 8% CO2. The strains were stored at ~70°C in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) containing 20% (vol/vol) glycerol (Sigma Chemical Co., St. Louis, Mo.). E. coli XL1-Blue MRF- (ΔmcrCB-λhsdSMR-mrr) endA1 supE44 thi-1 recA1 gyrA96 relA1 lac (F’ proAB lacIqZAM15 Tn10 [Tet’]) (Stratagene, La Jolla, Calif.) and E. coli B strain BL21 (F’ dcm ompT hsdS dE384 gyrA96 relA1 lac (F’ proAB lacIqZAM15 Tn10 [Tet’]) (Stratagene, La Jolla, Calif.) and E. coli B strain BL21 (F’ dcm ompT hsdS dE384 gyrA96 relA1 lac (F’ proAB lacIqZAM15 Tn10 [Tet’]) (Stratagene, La Jolla, Calif.) and E. coli B strain BL21) were grown on Lennox Luria-Bertani agar or broth (Gibco BRL, Gaithersburg, Md.) at 37°C. Where appropriate, 100 μg of ampicillin (Sigma) per ml was added to the medium. The low-copy-number plasmid pWK30 (41) and plasmid p629 (15) were used to clone the nspA gene as well as to produce the gonococcal NspA protein.

 Colony hybridization with an N. meningitidis nspA probe. A DNA probe was prepared by PCR amplification of the nspA gene from N. meningitidis 608B (28) with oligonucleotide primers NC-01 (5′-ATG AAA AAA GCA CTT GCC ACA CTG-3′) and NC-18 (5′-TCA GAA TTT GAC GCG CAC GCC G-3′) synthesized on an ABI synthesizer (Applied Biosystems, Inc., Mississauga, Canada). The amplification reactions were performed in 50-μl reaction mixtures containing 1 mM each primer, 100 ng of template genomic DNA of N. meningitidis 608B, and 2 U of Taq polymerase (Pharmacia Biotech, Baie d’Urfe, Canada). The samples were overlaid with 50 μl of mineral oil and subjected to 25 cycles of amplification consisting of denaturation at 94°C for 1 min, annealing at 65°C for 1 min, and polymerization at 72°C for 1 min. The 525-bp amplification product

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was purified by electrophoresis on a low-melting-point agarose gel and labeled by random priming with the DIG DNA labeling and detection kit (Boehringer Mannheim, Laval, Canada).

The purified recombinant bacterial strain to be tested were dotted onto a positively charged nylon membrane (Amersham Life Science, Oakville, Canada), dried, and then treated as specified by the manufacturer. Prehybridizations and hybridizations were done at 42°C with solutions containing 50% (vol/vol) formamide according to the DIG kit user’s guide for filter hybridization (Boehringer Mannheim). The prehybridization solution also contained 100 μg of denatured herring sperm DNA (Gibco BRL) per ml to prevent nonspecific hybridization of the DNA probe. The stringency washes and detection steps with the chemiluminescent reagent were also done as described in the DIG kit user’s guide.

Cloning and sequencing of the gonococcal nspA gene. A Southern hybridization (30) assay with a 3P-labeled DNA probe corresponding to a 2.75-kb ClaI fragment from the N. meningitidis genome with the nspA gene (26) was used for the identification of a 2.75-kb ClaI fragment containing the N. gonorrhoeae nspA gene. Briefly, chromosomal DNA from N. gonorrhoeae B2 was purified as described previously (26), digested with ClaI (Pharmacia Biotech), subjected to electrophoresis on an agarose gel, and transferred to a positively charged nylon membrane (Amersham Life Science). The N. meningitidis nspA probe was obtained by digestion of the plasmid pN2202 (28) and purification of the resulting 2.75-kb fragment with a QIAquick gel extraction kit (Qiagen Inc., Mountain View, Calif.). The DNA probe was labeled with [32P]dCTP (ICN Pharmaceuticals, Inc., Montreal, Canada) using the T7 Quick Prime Kit as specified by the manufacturer (Pharmacia Biotech). The probe DNA was denatured by heating to 95°C and allowed to cool to 60°C. The membrane was probed to a Kodak X-OMAT AR film (InterScience, Markham, Canada) with an intensifying screen. The N. gonorrhoeae ClaI fragments ranging from 2.5 to 3 kb that reacted positively to the nspA probe were purified from an agarose gel and were ligated into the low-copy-number plasmid pWKS30, which had been previously digested with ClaI and dephosphorylated. The recombinant plasmids were then transformed into E. coli XLI-Blue MRF as described by Hanahan (20). Colony hybridization was performed with the N. meningitidis nspA gene as a probe for the identification of positive transformants (36). Finally, positively hybridizing colonies were transferred onto a nitrocellulose membrane (Amersham). The M-5 MAb (27), which recognized the meningococcal NspA protein and is bactericidal for N. meningitidis (39) to identify clones producing the gonococcal nspA protein. The selected recombinant plasmid pWKS30 containing the gonococcal 2.75-kb insert was named pVR1. The nspA gene from N. gonorrhoeae was sequenced with the primers previously described for sequencing the meningococcal nspA gene (28). DNA sequencing was performed with the Taq Dye Terminator Cycle-sequencing kit and Applied Biosystems Inc. (Foster City, Calif.) automated sequencer model 373A as recommended by the manufacturer. A series of new primers were synthesized and used to obtain the sequence of the entire gene. Assembling and aligning of the sequence was done using the Sequencher software (Gene Codes Corp, Ann Arbor, Mich.) and Geneworks software (InfiniteGenetics, Inc, Mountain View, Calif.). The gonococcal nspA gene from strain B2 was amplified directly from purified chromosomal DNA by PCR with primers OCSB and OCSS (5'-GGT TCT GGC CAC GGG ATC ATG GAC TGG-3' and 5'-GGT TCT GGC CAC GGG ATC ATG GAC TGG-3'). The amplified DNA was cloned into the vector pCR2.1 (Invitrogen, San Diego, Calif.). The resulting plasmid pVR2 was sequenced with the primers previously described, and the resulting recombinant plasmid was identified as pVR24.

Production and purification of the N. gonorrhoeae NspA protein. Recombinant plasmids pURV1 and pURV24 were purified from E. coli XLI-Blue MRF transformants producing the NspA protein by using the Plasmid Midi kit (Qiagen Inc.) and transformed into BL21 transformed with plasmid pURV1 was inoculated in Lennox Luria-Bertani (LB) broth containing 100 μg of ampicillin per ml and was grown for 4 h at 37°C with agitation. Heat induction of expression of the nspA gene insert present on plasmid pURV24 was performed by the method described by George et al. (15). The bacterial cells were then removed from the cultures by centrifugation at 12,000 × g for 30 min at 4°C. The supernatant was filtered through a 0.22-μm pore-size filter and concentrated with an ultrafiltration apparatus and a Diaphlo ultrafiltration membrane YM 10 (Amicon Inc., Beverly, Mass.). The concentrated culture supernatant was subjected to 48% (vol/vol) ammonium sulfate precipitation before being fractionated by two successive chromatography steps with phenyl-Sepharose (Pharmacia) and cation-exchange Highload SP columns (Pharmacia). The purity of the NspA protein was shown to be ≥85% as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with the discontinuous buffer system of Laemmli (24) and staining with Coomassie brilliant blue.

Preparation of OM extracts. Lithium chloride extractions of the OM of N. gonorrhoeae B2 (12), OCRR-120 (5), OCRR-425, and 608B were performed as described previously (7). The protein concentrations were determined by the Lowry et al. method adapted to membrane fractions (25). Meningococcal and gonococcal OM preparations and NspA proteins were resolved by SDS-PAGE with 14% (vol/vol) polyacrylamide gels, and immunoblot analyses were performed as previously described.

Immunization of mice and generation of MAbs against the gonococcal NspA protein. BALB/c mice (Charles River Laboratories, Montreal, Canada) were injected subcutaneously three times at 3-week intervals with 20 μg of purified N. gonorrhoeae NspA protein in phosphate-buffered saline (PBS) containing 0.2 mg of OQPS (Geladane Labo, Honolulu, Hawaii). Sera were collected from each mouse at the period of fusion before each immunization and 2 weeks after the third injection. The sera were tested by enzyme-linked immunosorbent assay (ELISA) as described previously (19) with, as solid-phase antigens, gonococcal OM preparations (7.5 μg/ml) and gonococcal NpsA protein preparations (7.5 μg/ml), and purified gonococcal NspA protein (1 μg/ml). Three days before the fusion, the selected mouse was injected intravenously with 20 μg of gonococcal NspA protein. Hybridoma cell lines were produced as previously described (19). Supernatants collected from wells containing growing clones were tested for specific antibody production by ELISA. A second screening was performed on positive clones by Western immunoblotting (28) with the same protein preparations. Selected positive clones were grown by limiting dilution, expanded, and frozen in liquid nitrogen. The class, subclass, and light-chain type of MAbs were determined by ELISA with commercially available reagents (Southern Biotechnologies Associates Inc., Birmingham, Ala.).

Detection of the NspA protein in N. gonorrhoeae strains by whole-cell ELISA. To evaluate the reactivity of hybridoma supernatants against a panel of N. gonorrhoeae strains, whole-cell ELISA was used as previously described (1) with some modifications. Briefly, overnight gonococcal cultures were removed from chemically defined medium, washed, and suspended in PBS, and spread onto an optical density at 620 nm of OD<sub>620</sub> ≤0.6. Cell suspensions were aliquoted and frozen at −70°C until needed for the assay. The day before the assay, 0.1-ml portions of selected bacterial suspensions were dispensed into appropriate wells of a 96-well microtiter plate, which were allowed to microwave evaporate to dry. The wells were washed three times with PBS containing 0.02% (vol/vol) Tween 20 (J. T. Baker, Phillipsburg, N.J.) and blocked with 3% (wt/vol) bovine serum albumin (Sigma) in PBS per well. The plates were incubated for 30 min at 37°C, and the blocking solution was discarded. A 0.1-ml volume of hybridoma culture supernatant containing the NspA-specific MAb to be tested was added to each well, and the plates were incubated for 1 h at 37°C and then washed three times.

Alkaline phosphatase-conjugated AffiniPure Goat anti-mouse immunoglobulins (Jackson ImmunoResearch Laboratories, Mississauga, Canada) were diluted in PBS containing 0.5% (vol/vol) bovine serum albumin, and 0.1 μl of this solution was added to each well. The plates were incubated for 1 h at 37°C and washed three times with PBS containing 0.02% Tween 20, and 0.1 μl of n-phenylthiourea disodium phosphate solution in 10% diethanolamine (pH 9.6) was added to each well. Following incubation for 1 h at room temperature, the OD<sub>405</sub> was read with a Dynatech MRX microplate reader. Controls included wells containing cell culture medium without antibody and wells containing culture supernatant with unrelated MAbs. Values were considered positive when the OD<sub>405</sub> was greater than twice the reading obtained for control wells.

Radioimmunobinding assay. Binding of NspA-specific antibodies to whole gonococci was measured by a radioimmunobinding assay as previously described (2). Gonococcal and meningococcal strains were incubated with culture supernatant without antibody or culture supernatant containing MAbs specific to Haemophilus influenzae as negative controls. Results are expressed as means and standard deviation of counts per minute (cpm) obtained from the different experiments. Background values subtracted from all the binding values.

Nucleotide sequences. The nucleotide sequence of the gonococcal nspA gene described in this report has been assigned GenBank accession no. U52069. The nucleotide sequence of the N. meningitidis 608B gene is available from GenBank under the accession no. U52866. Contig 206 obtained from N. gonorrhoeae FA1000 is also available from the Gonococcal Genome Sequencing Project (B. A. Roe, S. P. Lin, L. Song, X. Yuan, S. Clifton, T. Ducey, L. Lewis, and D. W. Dyer), University of Oklahoma.

RESULTS

Distribution of the nspA gene in N. gonorrhoeae. A DNA probe derived from the N. meningitidis 608B nspA gene was used in colony hybridization assays to evaluate the distribution of nspA gene among gonococcal isolates. This nspA DNA probe was shown to hybridize with all 15 N. gonorrhoeae strains tested. These strains included eight serological reference strains and seven clinical isolates. As expected, the nspA probe also hybridized with the three N. meningitidis strains tested, which included one strain each of serogroups A, B, and C. The nspA probe did not hybridize with any of the 19 nonneisserial bacterial strains tested: Alcaligenes faecalis (ATCC 8750), Bordetella pertussis (9340), Bordetella bronchiseptica, Citrobacter freundii (ATCC 2080), Edwardsiella tarda.
FIG. 1. Predicted amino acid sequences of the NspA protein of N. gonorrhoeae B2, N. gonorrhoeae FA1090, and N. meningitidis 608B (28). Differences are indicated by the one-letter code, and identities are indicated by a period. Evaluation of the identities between these sequences indicated that these three proteins are highly similar, with a global identity of 93%. Sequence analysis revealed the presence of an additional amino acid residue (glutamine) at position 73 for the NspA protein from N. gonorrhoeae FA1090.

The NspA protein of Neisseria meningitidis 608B is shown in Fig. 1. The meningococcal NspA protein (28) that was previously shown to be cleaved in response to amino acids 1 to 19 (M-K-K-A-L-A-A-L-I-A-L-A-L-P-A-A-A-L), showed by using NspA-specific MAbs that the NspA protein was produced by the three clones. This result indicated the presence of a putative leader sequence corresponding to amino acids 1 to 19 (M-K-K-A-L-A-A-L-I-A-L-A-L-P-A-A-A-L-A) that was previously shown to be cleaved in the mature meningococcal NspA protein (28). Comparison of the deduced amino acid sequence of the NspA protein with the sequences compiled in GenBank and Swissprot revealed, as first observed for the meningococcal NspA protein, the existence of weak homology to Neisseria opacity proteins. The homology between the NspA protein and the Opa proteins was found to be clustered in two particular regions, located at the carboxy terminus and between amino acid residues 126 and 144 of the NspA protein.

An alignment of the two NspA deduced amino acid sequences derived from N. gonorrhoeae B2 and FA1090 and the sequence from N. meningitidis 608B is shown in Fig. 1. The NspA protein from strain FA1090 was obtained from the gonococcal genome-sequencing project, which is under way, and was found on contig 206. These three deduced polypeptide sequences are 93% identical. The level of identity increased to 98% when the two gonococcal sequences were compared, showing differences in only 3 positions out of 174 amino acid residues. In addition, the polypeptide from N. gonorrhoeae FA1090 has an insertion of one glutamine residue at position 73.

The molecular weight of the NspA polypeptide from N. meningitidis sequence was 18,316, and a predicted isoelectric point of 10.21 (Fig. 1). Analysis of the sequence also indicated the presence of a putative leader peptide sequence corresponding to amino acids 1 to 19 (M-K-K-A-L-A-A-L-I-A-L-A-L-P-A-A-A-L-A) that was previously shown to be cleaved in the mature meningococcal NspA protein (28). Comparison of the deduced amino acid sequence of the NspA protein with the sequences compiled in GenBank and Swissprot revealed, as first observed for the meningococcal NspA protein, the existence of weak homology to Neisseria opacity proteins. The homology between the NspA protein and the Opa proteins was found to be clustered in two particular regions, located at the carboxy terminus and between amino acid residues 126 and 144 of the NspA protein.

A preliminary alignment of the two NspA deduced amino acid sequences derived from N. gonorrhoeae B2 and FA1090 and the sequence from N. meningitidis 608B is shown in Fig. 1. The NspA protein from strain FA1090 was obtained from the gonococcal genome-sequencing project, which is under way, and was found on contig 206. These three deduced polypeptide sequences are 93% identical. The level of identity increased to 98% when the two gonococcal sequences were compared, showing differences in only 3 positions out of 174 amino acid residues. In addition, the polypeptide from N. gonorrhoeae FA1090 has an insertion of one glutamine residue at position 73.

The antigenic conservation of the gonococcal NspA protein. The mouse selected to generate the NspA-specific MAbs was immunized three times at 3-week interval with 20 μg of purified gonococcal rNspA protein mixed with 0.2 mg of Quil A per ml. This protein was immunogenic, since a high NspA-specific titer was recorded when the serum collected from this mouse was tested by ELISA against the rNspA protein. Screening of the hybridoma cell lines was performed by ELISA and immunoblotting with OM preparations from N. gonorrhoeae B2, N. meningitidis 608B, and the purified gonococcal rNspA protein. Seven NspA-specific hybridomas, secreting MAbs 11E9, 1D4, 144 of the NspA protein.

An alignment of the two NspA deduced amino acid sequences derived from N. gonorrhoeae B2 and FA1090 and the sequence from N. meningitidis 608B is shown in Fig. 1. The NspA protein from strain FA1090 was obtained from the gonococcal genome-sequencing project, which is under way, and was found on contig 206. These three deduced polypeptide sequences are 93% identical. The level of identity increased to 98% when the two gonococcal sequences were compared, showing differences in only 3 positions out of 174 amino acid residues. In addition, the polypeptide from N. gonorrhoeae FA1090 has an insertion of one glutamine residue at position 73.
NspA-specific MAbs. Four of the seven MAbs, namely, 11E9, 1D4, 13E5, and 5D1, reacted with all 51 *N. gonorrhoeae* strains tested, while MAbs 14D8, 14D7, and 2F10 recognized 50, 14, and 8 gonococcal strains, respectively. All of these MAbs recognized at least *N. meningitidis* 608B. This particular strain was previously shown to produce large amounts of NspA protein in the OM. MAbs 5D1, 14D7, and 2F10 reacted with all eight *N. meningitidis* strains. MAb 5D1 reacted with all meningococcal and gonococcal strains tested.

Immunoblot analysis clearly indicated that the protein produced in the culture supernatant of *E. coli* BL21 transformed with pURV1 was full-length and was reactive with the seven NspA-specific MAbs described in this report (Fig. 2A). As was the case for the meningococcal NspA protein, both the 22- and 18-kDa protein bands, which are known to be the two forms of the NspA protein that can be found after SDS-PAGE, were produced by the recombinant *E. coli* and were reactive with the NspA-specific MAbs. Figure 2B also shows the reactivity of MAbs 1D4, 5D1, and 11E9 with the native NspA protein present in OM preparations extracted from *N. gonorrhoeae* B2, MS11, and 5776. These MAbs also efficiently recognized in Western immunoblot analyses the two protein bands at 18 and 22 kDa, which are characteristic of the NspA protein after SDS-PAGE and were produced by *E. coli* BL21 transformed with pURV24, containing only the *nspA* gene insert (data not shown). This result clearly indicated that these MAbs recognized only the NspA protein and not other gonococcal proteins that might have been produced by plasmid pURV1.

**Surface exposure of the gonococcal NspA protein.** Radioimmunobinding assays were performed to determine whether the MAbs generated against the gonococcal NspA protein were able to recognize their specific epitopes at the surface of the gonococcus. MAbs specific to *Haemophilus influenzae* (P2-4 and 6B11) and hybridoma culture supernatant without anti-

### Table 1. Reactivity of the NspA-specific MAbs with different strains of *N. gonorrhoeae* and *N. meningitidis*

<table>
<thead>
<tr>
<th>MAb</th>
<th>Isotype</th>
<th>No. of strains recognized/total no. tested</th>
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<tr>
<td></td>
<td></td>
<td><em>N. gonorrhoeae</em></td>
</tr>
<tr>
<td>11E9</td>
<td>IgG1</td>
<td>51/51</td>
</tr>
<tr>
<td>1D4</td>
<td>IgG2a</td>
<td>51/51</td>
</tr>
<tr>
<td>13E5</td>
<td>IgG1</td>
<td>51/51</td>
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<tr>
<td>5D1</td>
<td>IgG2b</td>
<td>51/51</td>
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<td>14D8</td>
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<td>14D7</td>
<td>IgG1</td>
<td>14/51</td>
</tr>
<tr>
<td>2F10</td>
<td>IgG1</td>
<td>8/51</td>
</tr>
<tr>
<td>Me-7</td>
<td>IgG2a</td>
<td>9/51</td>
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</table>

* IgG, immunoglobulin G.
* Reactivity was considered positive when the ODs were greater than twice the reading obtained for control wells without antibody.
* *N. meningitidis* strains tested included two strains of serogroup B and one strain each of serogroups D, X, Z, A, Y, and W-135.

**FIG. 2.** Reactivity of the NspA-specific MAbs with recombinant and native NspA proteins. (A) SDS-PAGE migration profile of concentrated culture supernatant of recombinant *E. coli* BL21(pURV1) producing the gonococcal NspA protein (lane 2), and purified gonococcal rNspA protein (lane 3), and an immunoblot showing the reactivity of MAbs 11E9 (lane 1), 5D1 (lane 2), 1D4 (lane 3), 14D8 (lane 4), 13E5 (lane 5), 14D7 (lane 6), 2F10 (lane 7), and Me-7 (lane 8) with purified gonococcal recombinant NspA protein. (B) SDS-PAGE migration profile of OM preparations extracted from *N. gonorrhoeae* 5776 (lane 2), MS11 (lane 3), and B2 (lane 4), and an immunoblot showing the reactivity of MAb 11E9, 5D1, and 1D4 with these OM preparations. SDS-PAGE was performed with 14% polyacrylamide gels and the proteins were visualized by Coomassie blue staining. The first lane of each gel contained the pre-stained low-molecular-weight markers 43,000, 29,000, 18,400, 14,300, 6,200, 3,400, and 2,300 (Gibco BRL). The arrows indicate the locations of the NspA protein bands.
body were used as negative controls. Preliminary assays indicated that six of the eight MAbs tested, namely, 14D7, 1D4, 5D1, 12E12, 2F10, and Me-7 (anti-NspA of *N. meningitidis* 608B), bound strongly to their epitope at the surface of the gonococcus, with cpm values between 6,340 ± 393 and 23,649 ± 585 (data not shown). In these experiments, the background values were 572 ± 26 cpm when hybridoma culture supernatant without antibody was used as a negative control and 1,087 ± 307 cpm when bacteria were incubated with MAb 6B11. MAbs 11E9, 1D4, 5D1, and Me-7 were selected to conduct attachment study on additional strains. This panel of strains contained three gonococcal laboratory strains, one strain isolated from a patient with localized infection (strain 13-10), three strains isolated from patients with disseminated infection (strains 5776, 5517, and 3470) and the serogroup B meningococcal strain 608B. As shown in Fig. 3, high cpm values (between 8,861 ± 1,473 and 20,326 ± 10,525) were recorded for MAb 1D4, while MAb 11E9 attached only weakly at the surface of intact *N. gonorrhoeae* cells with cpm values between 1,159 ± 529 and 8,611 ± 4,171. Values obtained for MAb 5D1 were intermediate with respect to the other two MAbs tested. Background values were recorded when MAbs 11E9 and 1D4 were tested against meningococcal strain 608B, indicating that their corresponding epitopes are not exposed at the surface of intact meningococcal cells. The binding values obtained with these MAbs varied from one gonococcal strain to another. MAb Me-7, which reacted in whole-cell ELISA with only 9 of 51 gonococcal strains, attached efficiently at the surface of meningococcal strain 608B, with a mean cpm value of 24,955 ± 1,057. This MAb also recognized its specific epitope at the surface of intact gonococcal cells from strains B2 and 5776 but not the other strains.

**DISCUSSION**

We recently reported the identification of the NspA protein in the OM of *N. meningitidis* by using MAbs (27, 28). Some of these MAbs, such as Me-7, also reacted with certain gonococcal isolates, suggesting that a homologue of the meningococcal NspA protein could also be found in this closely related species.

Colony hybridization was performed with the *nspA* gene, which was originally cloned from the serogroup B meningococcal strain 608B as a probe to demonstrate the presence of an homologue of the *nspA* gene in the genome of *N. gonorrhoeae*. Strong hybridization signals were recorded with all 15 gonococcal and 3 meningococcal strains tested. This result indicated that the level of homology between the meningococcal *nspA* probe and a related sequence in the gonococcal genome was high enough to enable hybridization and detection. This was not surprising since most of the genes studied in either one of these bacterial species have a homologue in the other (38).

A strategy which yielded the first meningococcal *nspA* gene was adapted and successfully used to clone and sequence homologous gene from gonococcal strain B2. Interestingly, this *nspA* gene was found to be present on a 2.75-kb Clal genomic DNA fragment, as was the case for the meningococci. This is in accordance with what was previously reported, which indicated that the localization on the chromosome of most of the genes
residues. The NspA polypeptide from gonococcal strains were compared, they were determined to be deduced amino acid sequences of the NspA proteins from both present in the genome of gonococcal strain FA1090. When the reported (43).

Since the hybridization experiment suggested that the nspa gene was present in the genome of every gonococcal strains tested, we decided to generate additional Nspa-specific MAb to investigate the antigenic conservation of the gonococcal Nspa protein. As expected, whole-cell ELISA results clearly showed that the Nspa protein was produced by every gonococcal strain and that certain epitopes such as the ones recognized by MAb 11E9, 1D4, 13E5, and 5D1 were highly conserved (Table 1). Interestingly, all these MAb also recognized at least one of the eight meningococcal strains tested. In addition, MAb 5D1 was found to recognize every meningococcal and gonococcal strain tested. Similar findings were reported previously for another antigenically highly conserved outer membrane protein called Lip or H5 (10, 44). Indeed, MAb directed against this protein efficiently recognized N. gonorrhoeae as well as N. meningitidis strains but did not react with most nonpathogenic neisserial species. For this reason, it was suggested that this protein might be involved in pathogenesis. Unfortunately, no protective immunity was induced by this particular protein (4).

It was postulated that the failure of anti-Lip antibodies to promote bactericidal killing or to confer passive protection could be related to limited exposure of the antigen on the bacterial cells (35). To evaluate the surface exposure of the gonococcal Nspa protein, the attachment of the Nspa-specific MAb was evaluated by a radioimmunobinding assay. In contrast to what was observed for the Lip protein, the Nspa-specific MAb 1D4 and 5D1 efficiently recognized their corresponding epitopes at the surface of intact gonococcal cells, thus indicating that portions of the Nspa protein are exposed and easily accessible to the antibodies. Other epitopes of the gonococcal Nspa protein, such as the one recognized by MAb 11E9, seemed to be less accessible to the antibodies. The mouse used to generate the MAb was immunized with purified gonococcal rNspa protein. The conformation of the purified Nspa protein would not be expected to correspond exactly to the spatial organization of the native gonococcal Nspa protein when inserted in the OM. Interestingly, when tested by immunoblotting or whole-cell ELISA, these MAb also reacted well with the Nspa protein present in the OM of meningococcal strain 60SB. However, radioimmunobinding results indicated that their specific epitopes were not accessible at the surface of intact meningococcal cells (Fig. 3). This latter result suggested that the insertion of the Nspa protein is not exactly identical in these two species, thus directly affecting the attachment of the specific MAb, or that other components present at the surface of intact meningococcal cells might mask these epitopes, thus preventing the binding of the MAb.

Other epitopes on the meningococcal Nspa protein were shown to be easily accessible at the surface of intact meningococcal cells (unpublished data). In addition, MAb Me-7 was shown to be bactericidal against serologically distinct meningococcal strains, clearly confirming the notion that certain portions of the meningococcal Nspa protein are exposed at the cell surface (27).

In conclusion, we have shown that the gonococcal Nspa protein is closely related to its analogue, the meningococcal Nspa protein. This protein is present in the OM of all gonococcal strains tested so far, where it is easily accessible to specific antibodies. We are presently studying the possible role of the Nspa protein in the colonization of mucosal surfaces by the gonoccus.

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