Immune response to native NadA from Neisseria meningitidis and its expression in clinical isolates in Brazil

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A mAb against the NadA protein from Neisseria meningitidis strain 3006 (serosubtype B : 2b : P5.2,8) demonstrated strong bactericidal activity against Brazilian epidemic serogroup B strain N44/89 (B : 4,7 : P1.19,15 : P5.5,7) and a serogroup C strain, IMC 2135 (C : 2a : P1.5,2), but not against another serogroup C strain, N1002/90 (C : 2b : P1.3 : P5.8). The immunogenicity of native NadA in an outer-membrane vesicle (OMV) preparation was also tested. Serum from mice immunized with OMV from serogroup B strain N44/89, which contains the NadA protein, showed bactericidal activity against serogroup B and C strains possessing NadA. In dot-blot analysis of 100 serogroup B and 100 serogroup C isolates from Brazilian patients, the mAb to NadA recognized about 60% of the samples from both serogroups. The molecular mass of the NadA protein from strain N44/89 determined by mass spectrometry was 37 971 Da and the peptide sequences were identical to those of NadA from N. meningitidis strain MC58.

INTRODUCTION

Successful vaccines against Neisseria meningitidis serogroups A, C, Y and W135 offer protection in adults and in children over 2 years of age (Frasch, 1995). However, some recent studies have shown immunological hyporesponsiveness to a second vaccination (Granoff et al., 1998; Richmond et al., 2000). Improved vaccines composed of capsular polysaccharide–protein conjugate antigens have been investigated and these vaccines have been shown to be protective for younger children (Zollinger, 1997). Clinical trials have been performed with capsular polysaccharides from meningococcal serogroups A and C conjugated with the CRM197, a mutant diphtheria toxin (Anderson et al., 1994; Twumasi et al., 1995). Extension of this approach to a group B meningococcal vaccine is not straightforward, since the group B capsular polysaccharide is a poor immunogen in humans (Poolman, 1995). Development of vaccines against serogroup B has therefore focused mostly on the use of outer-membrane vesicles (OMV) containing different proteins (Blake & Gotschlich, 1986). Induction of protective immunity by these OMV vaccines has been studied in several trials and case-control studies. Trials in Chile and Brazil showed poor protection in children less than 4 years of age (Boslego et al., 1995; Milagres et al., 1994). Although efficacy in the range of 50–80% in children over 4 years old was recorded (Anderson et al., 1994), improvements are clearly needed.

Another limitation of OMV vaccines is that the protein antigens that induce bactericidal antibodies show sequence and antigenic variability. The bactericidal antibodies induced by OMV vaccines were found to be directed mainly against two classes of proteins, PorA (Poolman, 1995) and Opa (Rosenqvist et al., 1993). Therefore, interest has increased in finding conserved, surface-exposed proteins in serogroup B meningococcus that are able to induce bactericidal antibodies. NspA (Martin et al., 1997), GNA 33 (Pizza et al., 2000) and NadA (Comanducci et al., 2002), have been described as proteins that satisfy the requirements for broadly distributed surface proteins that induce bactericidal antibodies.
NadA is a novel, surface-exposed protein, which was described recently by Comanducci et al. (2002). It is an oligomeric protein with an apparent molecular mass of 190 kDa in SDS-PAGE. However, it was shown that the recombiant protein obtained by Comanducci et al. (2002) consists of 362 amino acids, including a possible leader peptide of 23 amino acids. The mature protein has a predicted molecular mass of 35363 Da, and a leucine zipper structure probably maintains its pentameric conformation.

In a previous report, sera of mice immunized with serogroup C strain IMC 2135 were shown to react with a protein of approximately 190 kDa in OMV from the serogroup B strain N44/89, as determined by immunoblotting (Fukasawa et al., 1999). This protein was therefore first studied as a 190 kDa monomer (Fukasawa et al., 2000). Furthermore, antibodies against this protein were found to be abundant in sera of Brazilian patients infected with serogroup B meningococcus (unpublished observations). The object of the present work was to characterize the antigenic properties of this protein further and to confirm its identity as NadA. In view of its potential importance as a component of meningococcal vaccines, the prevalence of this protein in strains isolated from Brazilian patients was also studied.

**METHODS**

**Bacterial strains.** The *N. meningitidis* strains N44/89 (B:4,7; C236), IMC 2135 (C:2a; P1.5,16b), and N1002/90 (C:2b; P1.3:P5.8) were used in these experiments. OMV proteins (20 μg) were separated by SDS-PAGE on gels containing 12% acrylamide and transferred electrophoretically to nitrocellulose paper. The transfer was performed at 250 mA for 1 h in a Bio-Rad Electroblotter, as specified by the manufacturer. After transfer, the nitrocellulose was blocked with BSA [3-0% (w/v) in PBS] for 30 min. The nitrocellulose was then incubated overnight with the serum samples diluted 1:50 in PBS-BSA. After washing four times, the strips were incubated with a peroxidase-conjugated goat anti-mouse IgG (Sigma) diluted 1:3000 in PBS-BSA. The nitrocellulose was washed three times with PBS and once with 0.05 M sodium phosphate, pH 5-0, and incubated for 30 min with hydrogen peroxide, 0.055% (v/v) (Aldrich), and 3-amino-9-ethylcarbazole, 4% (w/v) (Sigma), in 0.05 M sodium acetate, pH 5-5. The peroxidase reaction was stopped by washing the strips with water.

**RESULTS**

**Dot-blotting analysis.** Strains were tested by dot-blotting as described previously (Wedge et al., 1990). Briefly, whole-cell suspensions were dotted onto 0.45 μm pore-size nitrocellulose membrane. After drying for 30 min at room temperature, the membrane was blocked for 30 min with 10 ml BSA-PBS. Hybridoma culture supernatant was pipetted directly into blocking buffer at a final dilution of 1:10. After overnight incubation at room temperature on a rotator, the membrane was washed four times with PBS and incubated for 3 h with goat anti-mouse IgG conjugated to peroxidase (Sigma), diluted 1:3000. The membrane was then washed four times with PBS and developed with 3-amino-9-ethylcarbazole and hydrogen peroxide.

**Molecular mass determination and peptide sequencing.** De novo sequencing of peptides recovered from in-gel trypsin digestion of protein bands was achieved by liquid chromatography/tandem mass spectrometry (LC/MS/MS) on a Finnigan LCQ ion-trap mass spectrometer as described elsewhere (Shevchenko et al., 2002).
response. As shown in Fig. 1(a), mAb 1079B6 recognized a 170–200 kDa protein in OMV preparations from group B strains N44/98, 3006, M986 and MC58 and group C strain IMC 2135 in immunoblot assays. Fig. 1(b) shows the corresponding SDS-PAGE profile. OMV from strain N1002/90 did not possess this protein and the amount in OMV from strain MC58 was insufficient to be seen in SDS-PAGE.

Complement-mediated bactericidal activity of mAb 1079B6 was tested against strains N44/89, IMC 2135, 3006 and N1002/90. As shown in Table 1, high bactericidal titres were obtained against the three strains that contained the 190 kDa protein in OMV preparations but not against strain N1002/90, which lacked the protein.

In order to test whether the protein could induce cross-protection when present in the OMV, a group of mice were immunized with three doses of OMV vaccine prepared from strain N44/89. Serum obtained at 15 days after the third dose of this vaccine was used for bactericidal assay against two serogroup B and two serogroup C strains of different serotypes: N44/89 (B: 4,7 : P1.19,15 : P5.5,7), 3006 (B: 2b : P1.2 : P5.2,8), IMC 2135 (C: 2a : P1.5,2) and N1002/90 (C: 2b : P1.3 : P5.8) (Fig. 2). The vaccine induced bactericidal antibodies against the homologous strain and the other serogroup B strain 3006 and also against serogroup C strain IMC 2135, but not against the serogroup C strain N1002/90.

Molecular mass and partial peptide sequence of the 190 kDa protein

The monoisotopic mass calculated by LC/MS/MS was 37,931 Da. The peptide sequences obtained after trypsin hydrolysis, which covered about 77 % of the total protein, were aligned to the amino acid sequence of NadA from strain MC58 (Comanducci et al., 2002) and, over these regions, the sequences were identical (the amino acid sequence obtained is available as supplementary material in JMM Online at http://jmm.sgmjournals.org). Thus, the protein investigated in this report is presumed to be NadA.

Presence of the NadA protein in strains isolated from Brazilian patients

mAb 4895F9 was used to test by dot-blotting 100 group B and 100 group C strains isolated from Brazilian patients in 1999. Among the serogroup B strains tested, 36 % were serosubtype 4,7 : P1.19,15, which is the prevalent serosubtype found in Brazilian patients, and 80 % of these isolates bound the mAb in a dot-blot assay (Table 2). Therefore, overall, 62 % of the isolates were positive (Table 2). Therefore, overall, 62 % of the isolates were positive (Table 2). Therefore, overall, 62 % of the isolates were positive (Table 2). Therefore, overall, 62 % of the isolates were positive (Table 2).

DisCUSSION

A novel protein, NadA, was described by Comanducci et al. (2002) following a reverse vaccinology procedure (Pizza et
al., 2000). The NadA monomer has a molecular mass of about 35 kDa and forms very stable oligomers, probably pentamers. It is widely conserved among serogroup B and C strains. The recombinant protein induces bactericidal anti-pentamers. It is very important, since OMVs seem to be the best way to present antigens of meningococcus B.

About 60 % of serogroup B and C meningococcal isolates from Brazilian patients reacted with the mAb against NadA. This protein was present in 80 % of Brazilian strains of meningococcal serogroup B strains isolated in Brazil during 1997–1998, high prevalence of only three subtypes was found: P1.19,15 (66 %), P1.7,16 (11 %) and P1.7,16 (4 %) (Sacchi et al., 2001). NadA was shown to be present in 80 % of P1.19,15 strains, 40 % of P1.7,1 strains and 60 % of P1.7,16 strains (Table 2). The results of the cross-reactivity tests and screening of NadA described here are particularly important for the development of a vaccine for use in Brazil. The high prevalence of P1.19,15 strains and the high frequency of NadA in Brazilian strains may be used to define the serosubtype composition of an OMV vaccine against serogroup B to be used in Brazil. The present study suggests that an appropriate vaccine should contain an OMV preparation from serosubtype P1.19,15 expressing NadA.

ACKNOWLEDGEMENTS

This work was supported by FAPESP grant 00/08464-6 and PADCT/FINEP. Convenio 77.97.1152.00. L. O. F. is the recipient of a scholarship from FAPESP.

REFERENCES


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