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

Lucila O. Fukasawa,^{1,2} Maria Cecília O. Gorla,³ Ana Paula S. Lemos,³ Rocilda P. F. Schenkman,¹ Maria Cristina C. Brandileone,³ Jay W. Fox,⁴ Isaías Raw,¹ Carl E. Frasch⁵ and Martha M. Tanizaki¹

Correspondence
Martha M. Tanizaki
tanizaki@usp.br

¹Centro de Biotecnologia, Instituto Butantan, Avenida Vital Brasil 1500, CEP 05504-900, São Paulo, Brazil

²Curso de Pós-Graduação em Biotecnologia, USP-Butantan-IPT, Brazil

³Serviço de Bacteriologia, Instituto Adolfo Lutz, São Paulo, Brazil

⁴Department of Microbiology, University of Virginia, Charlottesville, VA, USA

⁵Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD, USA

A mAb against the NadA protein from *Neisseria meningitidis* strain 3006 (serosubtype B : 2b : P1.2 : 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.

Received 17 July 2002
Accepted 3 October 2002

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 ser-

ogroup 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.

Abbreviation: OMV, outer-membrane vesicle.

The NadA amino acid sequence obtained is available as supplementary material in JMM Online (<http://jmm.sgmjournals.org/>).

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 recombinant 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 35 363 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:P1.19,15:P5.5,7), IMC 2135 (C:2a:P1.5,2) and N1002/90 (C:2b:P1.3:P5.8) used in these experiments were isolated from Brazilian patients. Strains 3006 (B:2b:P1.2:P5.2,8) and M986 (B:2a:P1.5,2) were from the Center for Biologics Evaluation, FDA, Bethesda, USA, and strain MC58 (B:15:P1.7,16b) was provided by Dr Rino Rappuoli.

Immunization of mice. Male C3H/Hepas mice (Biotério de camundongos isogênicos, Inst. de Ciencias Biomédicas, Universidade de S. Paulo), 35 days old, were injected intraperitoneally with 0.5 ml of an OMV preparation of *N. meningitidis* N44/89 containing 17 µg protein and 25 µg aluminium hydroxide. A control group was injected with 0.5 ml saline containing 25 µg aluminium hydroxide. Mice were boosted with the same preparations at 14 and 28 days after the first immunization and bled via the retro-orbital plexus at 2 weeks after each injection.

Bactericidal activity. The bactericidal assay was carried out using Brazilian epidemic serogroup B strain N44/89 (B:4,7:P1.19,15:P5.5,7) and serogroup C strains N1002/90 (C:2b:P1.3:P5.8), IMC 2135 (C:2a:P1.5,2) and 3006 (B:2b:P1.2:P5.2,8) in 96-well plates, as described by Frasch & Robbins (1978), with modifications. Briefly, the final reaction mixture (50 µl) contained 25 µl of serial twofold dilutions of test serum that had been heat-inactivated at 56 °C for 30 min, 12.5 µl baby rabbit serum as the complement source, screened for absence of anti-meningococcal activity, and 12.5 µl of a suspension of exponential-phase meningococci (60 c.f.u. per well), grown in agar containing 1.0 % (v/v) horse serum. The reaction mixture was incubated at 37 °C for 30 min, and 130 µl tryptic soy agar (TSA), cooled to approximately 45 °C, was added to each well. The mixture was allowed to solidify and the plates were incubated for 18 h at 37 °C in 5 % CO₂. Viable counts were done at time 0 (*t*₀), before incubation with complement, in order to estimate the number of c.f.u. for 100 % survival, by plating 12.5 µl bacterial stock suspension onto a 150×150 mm TSA plate. Control wells included on each microtitre plate contained: (i) bacteria, complement and buffer (complement-dependent control) and (ii) bacteria,

heat-inactivated complement and buffer (complement-independent control). In addition, a positive control was included in each assay consisting of serial dilutions of a known positive serum. Assays were made in duplicate. Anti-meningococcal serogroup C bactericidal assays were carried out using Brazilian epidemic strains N1002/90 and IMC 2135 and performed as described above, except that the reaction was incubated at 37 °C for 60 min as standardized by Maslanka *et al.* (1997). The bactericidal titre of the serum was defined as the reciprocal of the serum dilution giving ≥50 % killing of the bacteria present at *t*₀.

SDS-PAGE and immunoblot analysis. SDS-PAGE and immunoblot detection of IgG antibodies against OMV from strain N44/89 were performed as described by Wedge & Froholm (1986) with modifications. 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 Electrobloetter, 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 acetate, pH 5.0, and incubated for 30 min with hydrogen peroxide, 0.05 % (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.

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.

mAbs. Murine mAbs were generated as described previously (Kearney *et al.*, 1979). Two groups of five BALB/c mice were immunized with three doses, each containing 5 µg OMV protein from strains 3006 or M986, at 2-week intervals. Four days after the last immunization, the spleens were removed and used for the fusion procedure. Positive clones were identified by ELISA using plates coated with the same OMV preparation used to immunize mice. Clones that bound the respective OMVs in ELISA were then examined by immunoblotting. mAb 1079B6 raised against strain 3006 and mAb 4895F9 raised against strain M986 OMV preparations were selected because they bound strongly on the immunoblots to a protein of approximately 170–200 kDa.

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).

RESULTS

Cross-reactivity among serogroup B and C strains

The mAbs 1079B6, raised against serogroup B strain 3006, and 4895F9, from serogroup B strain M986, were used to study the importance of the 190 kDa protein in the immune

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 1002/90 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

Fig. 1. Analysis of proteins in *N. meningitidis* OMV preparations. (a) Immunoblot showing binding of mAb 1079B6 to OMV preparations of strain N44/89 (lane 1), IMC 2135 (2), 3006 (3), M986 (4), MC58 (5) and N1002/90 (6). (b) SDS-PAGE profiles, after staining with Coomassie brilliant blue of OMV preparation from strains N44/89 (lane 1), IMC 2135 (2), 1002/90 (3) and MC58 (4). The arrow in (b) indicates a position equivalent to 200 kDa.

Table 1. Complement-mediated bactericidal activity of mAb 1079B6 against meningococcal strains

Strain	Titre
N44/89 (B: 4,7:P1.19,15:P5.5,7)	4000
IMC 2135 (C: 2a:P1.5,2)	8000
3006 (B: 2b:P1.2:P5.2,8)	3200
N1002/90 (C: 2b:P1.3:P5.8)	< 20

Fig. 2. Bactericidal activity of pooled mouse antiserum, obtained after immunization with an OMV preparation from *N. meningitidis* strain N44/89, against meningococcal strains serogroups B (N44/89, 3006) and C (IMC 2135, N1002/90). The bactericidal titres of the antiserum (open bars) and a saline control (filled bars) are shown.

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). Among serotype 4,7:P1.7,1 isolates, the second most prevalent group, only 40 % of the isolates were positive (Table 2). Therefore, overall, 62 % of the serogroup B strains were positive in the dot-blot assay. Among the serogroup C strains tested, 60 % of the strains were positive. Twenty per cent of serogroup B and C strains that were positive were tested in the immunoblot assay and, in all strains, the mAb reacted with a protein of 170–190 kDa (data not shown).

DISCUSSION

A novel protein, NadA, was described by Comanducci *et al.* (2002) following a reverse vaccinology procedure (Pizza *et*

Table 2. Dot-blot assay of Brazilian isolates of *N. meningitidis* serogroups B and C with mAb 4895F9 against NadA

Serosubtype	Strains (<i>n</i>)	Positive strains	
		<i>n</i>	%
Serogroup B			
2a : P1.5,2	4	4	100
4,7 : P1.7,1	10	4	40
4,7 : P1.13	3	1	33
4,7 : P1.19,15	36	29	80
4,10 : P1.9	2	1	50
10 : P1.15	3	3	100
15 : P1.7,16	5	3	60
19 : P1.15	4	1	25
19,7 : P1.15	2	2	100
19,14 : P1.22-1,14	4	1	25
Others	27	13	48
Totals	100	62	62
Serogroup C			
2a : P1.5,2	8	7	88
2b : P1.5,2	9	8	89
2b : P1.3	18	17	94
2b : P1.7,1	3	2	67
2b : P1.10	7	6	86
4,7 : P1.15	8	4	50
15 : P1.7,16	2	1	50
Others	45	15	33
Totals	100	60	60

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 antibodies (Comanducci *et al.*, 2002). In the present study, native OMV from strains containing NadA were used instead of the recombinant protein.

Pizza *et al.* (2000) identified a total of 350 candidate vaccine antigens in meningococcus serogroup B through whole-genome sequencing. Most of them are less abundant than the major outer-membrane proteins (Frasch *et al.*, 1985) and that is probably the reason that they have not been studied before. Due to its high apparent molecular mass, NadA is clearly visible in SDS-PAGE. The protein was seen in the OMV preparation of strain N44/89, the most prevalent serogroup B serosubtype in Brazil, and in strain 3006, as well as in the serogroup C strain IMC 2135, which is used in Brazil for production of the serogroup C capsular polysaccharide. However, in strain MC58, from which the recombinant NadA was obtained, the protein seemed to be less abundant, since it was not visible in the protein profile of OMV.

NadA is an immunogenic and conserved protein. The mAb 1079B6, raised against NadA in an OMV preparation from a serogroup B strain, gave a strong bactericidal reaction against

homologous and heterologous strains. Furthermore, NadA apparently induces cross-reactivity from the OMV preparations. Bactericidal activity of a pooled serum from mice immunized with OMV from strain N44/89 was found against strains IMC 2135 and 3006, but not against strain N1002/90, which was negative for NadA by immunoblotting. Much of this bactericidal cross-reactivity could be attributed to the 190 kDa protein (NadA), since these vaccine strains have different class 1 (PorA) and class 5 (Opa) proteins. This result 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 serosubtype B : 4,7 : P1.19,15. This particular serosubtype is the most prevalent in Brazil and accounts for 66 % of all serotyped meningococcal serogroup B isolates (Sacchi *et al.*, 2001). However, it is not clear whether the dot-blot analysis is sufficiently sensitive to detect all NadA-positive strains.

Among the meningococcal B strains isolated in Brazil during 1997–1998, high prevalence of only three subtypes was found: P1.19,15 (66 %), P1.7,1 (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

- Anderson, E. L., Bowers, T., Mink, C. M., Kennedy, D. J., Belshe, R. B., Harakeh, H., Pais, L., Holder, P. & Carlone, G. M. (1994). Safety and immunogenicity of meningococcal A and C polysaccharide conjugate vaccine in adults. *Infect Immun* **62**, 3391–3395.
- Blake, M. S. & Gotschlich, E. C. (1986). Functional and immunological properties of pathogenic *Neisseria* surface proteins. In *Bacterial Outer Membranes as Model Systems*, pp. 377–400. Edited by M. Inoue. New York: Wiley.
- Boslego, J., Garcia, J., Cruz, C. & 11 other authors (1995). Efficacy, safety, and immunogenicity of a meningococcal group B (15:P1.3) outer membrane protein vaccine in Iquique, Chile. Chilean National Committee for Meningococcal Disease. *Vaccine* **13**, 821–829.
- Comanducci, M., Bambini, S., Brunelli, B. & 12 other authors (2002). NadA, a novel vaccine candidate of *Neisseria meningitidis*. *J Exp Med* **195**, 1445–1454.
- Frasch, C. E. (1995). Meningococcal vaccines. In *Meningococcal Disease*, pp. 245–283. Edited by K. Cartwright. New York: Wiley.

- Frasch, C. E. & Robbins, J. D. (1978). Protection against group B meningococcal disease. III. Immunogenicity of serotype 2 vaccines and specificity of protection in a guinea pig model. *J Exp Med* **147**, 629–644.
- Frasch, C. E., Zollinger, W. D. & Poolman, J. T. (1985). Serotype antigens of *Neisseria meningitidis* and a proposed scheme for designation of serotypes. *Rev Infect Dis* **7**, 504–510.
- Fukasawa, L. O., Gorla, M. C. O., Schenkman, R. P. F., Garcia, L. R., Carneiro, S. M., Raw, I. & Tanizaki, M. M. (1999). *Neisseria meningitidis* serogroup C polysaccharide and serogroup B outer membrane vesicle conjugate as a bivalent meningococcus vaccine candidate. *Vaccine* **17**, 2951–2958.
- Fukasawa, L. O., Gorla, M. C. O., Lemos, A. P., Brandileone, M. C., Schenkman, R. P. F., Frasn, C. E., Raw, I. & Tanizaki, M. M. (2000). A 190 kDa conserved surface protein from *Neisseria meningitidis* that induces bactericidal antibodies. In *Abstract Book of the Twelfth International Pathogenic Neisseria Conference*, Galveston, TX, USA.
- Granoff, D. M., Gupta, R. K., Belshe, R. B. & Anderson, E. L. (1998). Induction of immunologic refractoriness in adults by meningococcal C polysaccharide vaccination. *J Infect Dis* **178**, 870–874.
- Kearney, J. F., Radbruch, A., Liesegang, B. & Rajewsky, K. (1979). A new mouse myeloma cell line that has lost immunoglobulin expression but permits the construction of antibody-secreting hybrid cell lines. *J Immunol* **123**, 1548–1550.
- Martin, D., Cadieux, N., Hamel, J. & Brodeur, B. R. (1997). Highly conserved *Neisseria meningitidis* surface protein confers protection against experimental infection. *J Exp Med* **185**, 1173–1183.
- Maslanka, S. E., Gheesling, L. L., Libutti, D. E. & 14 other authors (1997). Standardization and a multilaboratory comparison of *Neisseria meningitidis* serogroup A and C serum bactericidal assays. The Multilaboratory Study Group. *Clin Diagn Lab Immunol* **4**, 156–167.
- Milagres, L. G., Ramos, S. R., Sacchi, C. T., Melles, C. E., Vieira, V. S., Sato, H., Brito, G. S., Moraes, J. C. & Frasn, C. E. (1994). Immune response of Brazilian children to a *Neisseria meningitidis* serogroup B outer membrane protein vaccine: comparison with efficacy. *Infect Immun* **62**, 4419–4424.
- Pizza, M., Scarlato, V., Masignani, V. & 33 other authors (2000). Identification of vaccine candidates against serogroup B meningococcus by whole-genome sequencing. *Science* **287**, 1816–1820.
- Poolman, J. T. (1995). Development of a meningococcal vaccine. *Infect Agents Dis* **4**, 13–28.
- Richmond, P., Kaczmarek, E., Borrow, R., Findlow, J., Clark, S., McCann, R., Hill, J., Barker, M. & Miller, E. (2000). Meningococcal C polysaccharide vaccine induces immunologic hyporesponsiveness in adults that is overcome by meningococcal C conjugate vaccine. *J Infect Dis* **181**, 761–764.
- Rosenqvist, E., Hoiby, E. A., Wedege, E., Kusecek, E. & Achtman, M. (1993). The 5C protein of *Neisseria meningitidis* is highly immunogenic in humans and induces bactericidal antibodies. *J Infect Dis* **167**, 1065–1073.
- Sacchi, C. T., Lemos, A. P., Popovic, T. & 8 other authors (2001). Serosubtypes and PorA types of *Neisseria meningitidis* serogroup B isolated in Brazil during 1997–1998: overview and implications for vaccine development. *J Clin Microbiol* **39**, 2897–2903.
- Shevchenko, A., Chernushevic, I., Shevchenko, A., Wilm, M. & Mann, M. (2002). ‘De novo’ sequencing of peptides recovered from in-gel digested protein by nanoelectrospray tandem mass spectrometry. *Mol Biotechnol* **20**, 107–118.
- Twumasi, P. A., Jr, Kumah, S., Leach, A. & 9 other authors (1995). A trial of a group A plus group C meningococcal polysaccharide-protein conjugate vaccine in African infants. *J Infect Dis* **171**, 632–638.
- Wedge, E. & Froholm, L. O. (1986). Human antibody response to a group B serotype 2a meningococcal vaccine determined by immunoblotting. *Infect Immun* **51**, 571–578.
- Wedge, E., Hoiby, E. A., Rosenqvist, E. & Froholm, L. O. (1990). Serotyping and subtyping of *Neisseria meningitidis* isolates by co-agglutination, dot-blotting and ELISA. *J Med Microbiol* **31**, 195–201.
- Zollinger, W. D. (1997). New and improved vaccines against meningococcal disease. In *New Generation Vaccines*, pp. 469–488. Edited by M. M. Levine, G. C. Woodrow, J. B. Kaper & G. S. Cobon. New York: Marcel Dekker.