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Solicitud de Patente de Modelo de utilidad para:

**"VACUNAS PARA PROTECCION DE ESPECTRO AMPLIO CONTRA
ENFERMEDADES CAUSADAS POR NEISSERIA MENINGITIDIS"**

Solicitante: CHILDREN'S HOSPITAL & RESEARCH CENTER AT OAKLAND.

RESPUESTA AL EXAMEN DE BUSQUEDA DE ANTERIORIDADES

MARGARITA CASTELLANOS ABONDANO, mayor de edad, identificada y domiciliada como aparece al pie de mi firma, en atención a su oficio No. 2816 Notificado el 2 de Marzo de 2007, atentamente me permito dar repuesta al mismo de la siguiente manera:

Presento copia completa de los artículos requeridos por ese Despacho como resultado de la búsqueda de anterioridades:

- x] TAPPERO JORDAN W ET AL: "Immunogenicity of 2 serogroup B outer-membrane protein meningococcal vaccines: A randomized controlled trial in Chile" JAM (JOURNAL OF THE AMERICAN MEDICAL ASSOCIATION), vol. 281, No. 16, 28 April 1999 (1999-04-28), pages 1520-1527, XP009022137 ISSN: 0098-7484.
- X] RUPPE VAN DER VOORT E ET AL: "Immunogenicity studies with a genetically engineered hexavalent PorA and a wild-type meningococcal group B outer membrane vesicle vaccine in infant cynomolgus monkeys." VACCINE. 31 JAN 2000, vol. 18, no. 14, 31 January 2000 (2000-01-31), pages 1334-1343, XP002314869 ISSN: 0264-410X.

- MOE GREGORY R ET AL: "Differences in surface expression of NspA among *Neisseria meningitidis* group B strains" INFECTION AND IMMUNITY, vol. 67, no. 11, November 1999 (1999-11), pages 5664-5675, XP002266209 ISSN: 0019-9567.
- TJ MOE G R ET AL: "Functional activity of anti-neisserial surface protein A monoclonal antibodies against of *Neisseria meningitidis* serogroup B" INFECTION AND IMMUNITY, vol. 69, no.6, June 2001(2001-06), pages 3762-3771, XP002314871 ISSN: 0019-9567.
- DEVI S J ET AL: "Binding diversity of monoclonal antibodies to alpha (2→8) polysialic acid conjugated to outer membrane vesicle via adipic acid dihydrazide." FEMS IMMUNOLOGY AND MEDICAL MICROBIOLOGY. JUL 1996, vol. 14, no. 4, July 1996 (1996-07), pages 211-220, XP002315243 ISSN: 0928-8244.

Cumplido así con lo dispuesto, atentamente solicito a Ud. se continúe con el trámite correspondiente a esta solicitud de Patente de Invención.

De la Señora Jefe,


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CLINICAL INVESTIGATION

Immunogenicity of 2 Serogroup B Outer-Membrane Protein Meningococcal Vaccines

A Randomized Controlled Trial in Chile

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MENINGOCOCCAL DISEASE caused predominantly by *Neisseria meningitidis* serogroups A, B, and C occurs predominantly in young children and remains a substantial cause of morbidity and mortality worldwide.^{1,2} In addition to causing endemic disease globally, meningococci, unlike other encapsulated bacteria, cause epidemics. Serogroup B epidemics, problematic in Norway and throughout much of Latin America in the 1980s and 1990s,³ have recently emerged in New

See also pp 1493 and 1541 and Patient Page.

Context Meningococcal disease occurs worldwide, and serogroup B disease accounts for a large proportion of cases. Although persons younger than 4 years are at greatest risk for serogroup B meningococcal disease, vaccine efficacy has not been demonstrated in this age group.

Objective To evaluate serum bactericidal activity (SBA) against homologous vaccine type strains and a heterologous Chilean epidemic strain of *Neisseria meningitidis* as a potential correlate for vaccine efficacy.

Design Double-blind, randomized controlled trial conducted between March 14 and July 20, 1994. All blood samples were taken by December 1994.

Setting Santiago, Chile, where a clonal serogroup B meningococcal disease epidemic began in 1993.

Participants Infants younger than 1 year (n = 187), children aged 2 to 4 years (n = 183), and adults aged 17 to 30 years (n = 173).

Intervention Participants received 3 doses of outer-membrane protein (OMP) meningococcal vaccine developed in either Cuba or Norway or a control vaccine, with each dose given 2 months apart. Blood samples were obtained at baseline, prior to dose 3, and at 4 to 6 weeks after dose 3.

Main Outcome Measure Immune response, defined as a 4-fold or greater rise in SBA titer 4 to 6 weeks after dose 3 compared with prevaccination titer.

Results Children and adult recipients of either meningococcal vaccine were more likely than controls to develop an immune response to the heterologous epidemic strain. After 3 doses of vaccine, 31% to 35% of children responded to the vaccine vs 5% to placebo; 37% to 60% of adults responded to vaccine vs 4% to placebo (P < .05 vs control for all). Infants, however, did not respond. In contrast, against homologous vaccine type strains, the response rate was 67% or higher among children and adults and 90% or higher among infants (P < .001 vs control for all). Subsequent SBA against 7 isogenic homologous target strains identified class 1 OMP as the immunodominant antigen.

Conclusions These data suggest that neither serogroup B OMP meningococcal vaccine would confer protection during a heterologous epidemic. However, epidemic strain-specific vaccines homologous for class 1 OMP are promising candidates for the control of epidemic serogroup B meningococcal disease.

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Zealand⁴ and the United States.⁴⁻⁶ Response to serogroup B epidemics, unlike serogroup A and C epidemics, is difficult because existing serogroup B vaccines have not been shown to be efficacious on an international scale.⁷⁻¹⁰

Quadrivalent meningococcal polysaccharide vaccine is efficacious against meningococcal disease caused by the A, C, W-135, and Y serogroups.¹¹⁻¹³ Serogroup B polysaccharide antigen,

however, is poorly immunogenic in humans,^{14,15} and the elicitation of antibodies to serogroup B polysaccharide antigen is of concern because this antigen is present in human neonatal neural tissue.^{16,17} Therefore, alternative

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strategies for serogroup B meningococcal vaccine development focusing on outer-membrane protein (OMP) antigens have been pursued.¹⁷⁻¹⁹

The serum bactericidal activity (SBA) assay has been shown to be the most important serologic correlate for vaccine efficacy.²⁰⁻²² Serum bactericidal activity has also become the primary serologic assay used to assess protective immunity stimulated by serogroup B meningococcal vaccine candidates.^{10,23-26} Recent evidence suggests that class 1 OMP, encoded by the *porA* gene,²⁷ plays a major role in the SBA immune response following meningococcal carriage,²⁸ invasive disease,²⁹ and immunization with a serogroup B OMP meningococcal vaccine.²⁷⁻²⁹

During the mid-1980s and 1990s, the city of Iquique, Chile, experienced high incidence rates (20-35 per 100 000) of serogroup B meningococcal disease.³⁰ In 1993, a sharp increase in incidence (5.9 per 100 000) was recognized in metropolitan Santiago, Chile; more than 60% of cases occurred among children younger than 5 years.³¹ The predominant serogroup B strain in Santiago was identical to the strain causing endemic disease in Iquique. To control the expanding Chilean epidemic, 2 OMP-based vaccines against serogroup B meningococcal disease, one produced by the Finlay Institute (FI) in Havana, Cuba, and another by the National Institute of Public Health (NIPH) in Oslo, Norway, were considered for widespread vaccination. Vaccine efficacy had been demonstrated among children (age range, 10-16 years) in double-blind, randomized controlled trials using 2-dose regimens of the FI-produced vaccine in Cuba and the NIPH-produced vaccine in Norway (estimated vaccine efficacy, 83% after 16 months and 57% after 29 months, respectively).¹⁴ However, vaccine efficacy was not evaluated in younger children in these initial trials. In 1989 and 1990, the FI-produced vaccine was used to control a serogroup B meningococcal epidemic in São Paulo, Brazil; in a 2-dose regimen, vaccine efficacy was not demonstrated among children younger than 4 years.⁹

METHODS

Study Design

We conducted a prospective, double-blind, randomized controlled comparison trial of immunogenicity and reactogenicity elicited by 2 serogroup B OMP meningococcal vaccines among 3 age groups of Santiago residents, including infants (younger than 1 year), children (aged 2-4 years), and adults (aged 17-30 years). Infants and children were recruited from among healthy Consultorio Maternal clinic attendees and adults from San José Hospital staff. Sample sizes were designed to include 165 participants per age group, with one third of the participants in each age group randomized to receive the FI-produced vaccine, the NIPH-produced vaccine, or a control vaccine. In each age group, sequentially enrolled subjects were assigned 1 of 6 unique study group numbers. Two numbers were randomly assigned to each of the 3 possible vaccine groups. With the exception of 1 staff member assigned to draw vaccines, all investigators and study participants were blinded to vaccine study group numbers until completion of the study.

Before enrollment, written informed consent was obtained from parents of participating children and from adult study participants, as approved by the Chilean Ministry of Health and the Human Subjects Protection Institutional Review Board of the Centers for Disease Control and Prevention (CDC).

Vaccines and Clinical Specimens

The FI-produced vaccine consisted of lipooligosaccharide-reduced outer membrane vesicles from an epidemic Cuban strain of *N meningitidis* (CU385, B:4:P1.15); each dose contained 30 µg of OMP and 50 µg of serogroup C meningococcal polysaccharide.¹⁴ The NIPH-produced vaccine consisted of lipooligosaccharide-reduced outer-membrane vesicles from a Norwegian epidemic strain of *N meningitidis* (44/76, B:15:P1.7,16); each dose contained 25 µg of OMP, with no detectable meningococcal polysaccharide.^{7,32}

Both vaccines present their partially rifled OMP as protoliposome vesicles sorbed to aluminum hydroxide. Vaccines were provided by their respective manufacturers to the Chilean Ministry of Health and stored at 4°C until the day of vaccination.

Vaccines were administered intramuscularly as a 3-dose regimen, with each dose given 2 months apart. Infant and child control groups received a 3-dose series of a licensed *Haemophilus influenzae* type b (Hib) polyribosyl ribitol phosphate tetanus conjugate vaccine (Pasteur Merieux, Paris, France); adult controls received 3 doses of a minium hydroxide adjuvant diphtheria in solvent. Following unblinding at completion of the study, Hib vaccine was offered to all infants and children who had received a serogroup B meningococcal vaccine.

Blood samples were obtained from each study participant prior to vaccination, prior to dose 3, and approximately 4 to 6 weeks following the third dose of vaccine.

Immunogenicity

Serum bactericidal activity assays were conducted by standard methods.^{23,33} Vaccine response was defined as a 4-fold or greater rise in SBA antibody titer compared with prevaccination titer; response was defined as less than 4-fold rise in titer.

All *N meningitidis* target strains were immunologically classified by serogroup (capsular polysaccharide), serotype (class 2 or 3 OMP), subtype (class 1 OMP), and immunotype (lipooligosaccharide) using standard nomenclature (serogroup, serotype, subtype, and immunotype, respectively, separated colons).³⁴ In addition, DNA sequence data for *porA* gene variable regions 1 and 2 are available in the GenBank for the Chilean epidemic strain CH5 (AF051536), FI-produced vaccine type strain CU385 (U92935), and NIPH-produced vaccine type strain 44/76 (X52995). Quality control of target strains was conducted periodically throughout laboratory testing by monoclonal antibody reactivity.³⁵

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Three serogroup B *N meningitidis* strains were used in the testing of serum samples prior to the unblinding of study group numbers: the Chilean epidemic strain (CH539, B:15:P1.3:L3,7,9; DNA sequence subtype P1.7³), the FI-produced vaccine type strain (CU385, B:4:P1.15:L3,7,9; DNA sequence subtype P1.19,15), and the NIPH-produced vaccine type strain (44/76, B:15:P1.7,16:L3,7,9; DNA sequence subtype P1.7,16). These 3 strains are members of a genetically distinct complex (enzyme type 5 [ET-5]) of *N meningitidis* clones.^{27,28} Multilocus enzyme electrophoresis testing of these 3 strains gave identical results with a panel of 24 enzymes.²⁹

Vaccine type strains were considered homologous during testing of serum samples from participants who were vaccinated with the vaccine based on that strain (eg, CU385 is homo-

logous for a person vaccinated with the FI-produced vaccine). Other comparisons were considered heterologous (eg, CU385 and CH539 are heterologous for a participant vaccinated with the NIPH-produced vaccine; 44/76 and CH539 are heterologous for a participant vaccinated with the FI-produced vaccine). Because neither the FI-produced nor NIPH-produced vaccine is based on a Chilean epidemic strain, the Chilean strain was considered heterologous in all assays performed.

To identify possible immunodominant antigens responsible for homologous SBA, a set of 7 isogenic strains was constructed from the NIPH-produced vaccine type strain (44/76, B:15:P1.7,16); these strains appear to differ only in their OMP compositions.^{24,29} Following unblinding of study group numbers, SBA assays were conducted on samples of infant serum obtained

from recipients of NIPH-produced vaccine using the following 7 genetically manipulated serogroup B target strains: a class 1 OMP-deficient strain (B:15-); a class 3 OMP-deficient strain (B:P1.7,16); and strains B:15:P1.5,2; B:15:P1.19,15; B:15:P1.7⁴,4; B:15:P1.12,13; and B:15:P1.5⁴,10.

All SBA assays using the Chilean epidemic strain and the 7 isogenic strains as target strains were performed at the CDC. Serum bactericidal activity assays using the vaccine type strains as target strains were performed both at the CDC and NIPH. Human complement lots used at the CDC (lot 1-27-93) and the NIPH (lot HH) were shown to be comparable; both lots lacked SBA against study target strains. Results were highly concordant between the 2 laboratories.

Safety Monitoring

Reactogenicity was systematically monitored by a study nurse who conducted daily home visits following each dose of vaccine for a minimum of 7 days or until all vaccine-related adverse effects had resolved. Although clinical symptoms were quantified as being absent or present with moderate or severe interference with normal activity, they were analyzed as being either absent or present, regardless of level of normal activity interference.

Statistical Analysis

All data were entered into standardized electronic databases with Epi Info software; analyses were performed with Epi Info and SAS software.^{40,41} The Kruskal-Wallis test and the χ^2 test were used to compare distributions of continuous variables and categorical variables, respectively.

RESULTS

Prevaccination blood samples were obtained from 187 infants, 183 children, and 173 adults. At enrollment, the mean ages of infants (7.1 months), children (3.0 years), and adults (23.3 years) were similar for FI-produced, NIPH-produced, and control vaccine recipients (TABLE 1). There was no significant difference in sex distribution or

Table 1. Characteristics of Participants by Study Age Group and Vaccine Received*

Characteristic	Vaccine			P Value†
	FI-Produced	NIPH-Produced	Control	
Infants (Aged <1 y)				
	(n = 68)	(n = 68)	(n = 68)	
Age, mean, mo	3.8	4.1	4.3	.33
Sex, female, %	55	57	59	.81
Time from sample 1 to sample 2, mean (range), d‡	110 (85-135)	110 (85-150)	110 (85-141)	>.99
Time from sample 1 to sample 3, mean (range), d‡	143 (88-184)	142 (101-182)	144 (117-174)	.88
Children (Aged 2-4 y)				
	(n = 83)	(n = 81)	(n = 88)	
Age, mean, y	2.5	2.5	3.1	.71
Sex, female, %	47	49	45	.75
Time from sample 1 to sample 2, mean (range), d‡	113 (104-125)	113 (103-128)	113 (101-129)	.88
Time from sample 1 to sample 3, mean (range), d‡	148 (117-187)	148 (118-187)	148 (118-183)	.98
Adults (Aged 17-90 y)				
	(n = 68)	(n = 67)	(n = 67)	
Age, mean, y	22.9	23.7	23.2	.49
Sex, female, %	71	65	61	.07
Time from sample 1 to sample 2, mean (range), d‡	109 (70-123)	111 (103-133)	109 (75-120)	.94
Time from sample 1 to sample 3, mean (range), d‡	150 (135-211)	150 (138-189)	149 (135-178)	.48

†FI indicates Finlay Institute; NIPH, National Institute of Public Health. Infant and child controls received *Haemophilus influenzae* type B conjugate vaccine; adult controls received aluminum hydroxide adjuvant diphtheria toxoid. All vaccines were administered intramuscularly on a 0-clone regimen, with each dose given 2 months apart.

‡Data were calculated using the Kruskal-Wallis test or the χ^2 test.

§Blood samples were obtained prior to vaccination (sample 1) and approximately 4 weeks following the second (sample 2) and 4 to 6 weeks following the third (sample 3) doses of vaccine.

*FI, Instituto Finlay Institute; NIPH, National Institute of Public Health. Infant and child controls received *Haemophilus influenzae* type b conjugate vaccine; adult controls received aluminum hydroxide adjuvant diphtheria toxoid. All vaccines were administered intramuscularly as a 3-dose regimen, with each dose given 2 months apart.

†Data were calculated using the Kruskal-Wallis test or the χ^2 test.
‡Blood samples were obtained prior to vaccination (sample 1) and approximately 4 weeks following the second (sample 2) and 4 to 6 weeks following the third (sample 3) doses of vaccine.

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time from blood sample 1 to subsequent blood samples by age group among the 3 vaccine recipient groups (Table 1). Seven infants (4%), 18 children (10%), and 18 adults (10%) withdrew from the study (FIGURE). Inadequate volume of serum samples precluded some SBA assays in a small proportion of participants (TABLE 2). There was no significant difference in numbers withdrawn or excluded because of inadequate serum samples by age group among the 3 vaccine recipient groups ($P>.05$ for all).

Immunogenicity

Chilean Epidemic Strain. Among infants, there was no significant difference in SBA response to the heterologous Chilean epidemic target strain (CH539; B:15:P1.7^h,3) between those vaccinated with either the FI-produced or NIPH-produced vaccine and those vaccinated with the Hib control vaccine (Table 2). Among both children and adults, recipients of either meningococcal vaccine were more likely than recipients of control vaccine to respond ($P<.002$ vs control for all). Only among adults was response between NIPH-produced vaccine recipients (blood sample 3, 60%) and FI-produced vaccine recipients (blood sample 3, 37%) significantly different ($P=.03$).

Vaccine Type Strains. For all 3 age groups, recipients of the FI-produced and NIPH-produced vaccines were more likely to mount antibody responses against their respective homologous vaccine type strains than were recipients of control vaccine ($P<.001$ vs control for all) (Table 2). For all 3 age groups, recipients of FI-produced vaccine were more likely than recipients of NIPH-produced vaccine to respond against the FI-produced vaccine type strain (blood sample 3 for infants and children, $P<.001$; blood sample 3 for adults, $P=.05$). For all 3 age groups, recipients of NIPH-produced vaccine were more likely than recipients of FI-produced vaccine to respond against the NIPH-produced vaccine type strain (blood sample 3, $P<.001$ for all).

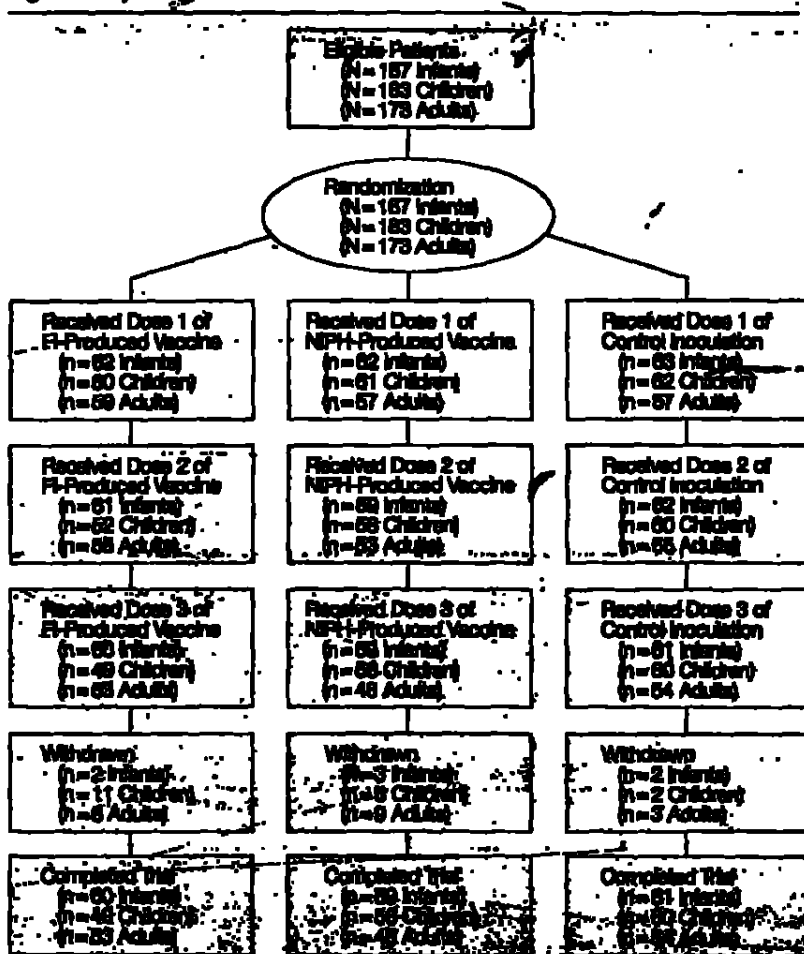
Isogenic Strains. At blood sample 3, 52 (98%) of 53 infants vaccinated with the NIPH-produced vaccine showed a 4-fold or greater rise in antibody titer against the homologous NIPH-produced vaccine type strain devoid of class 3 OMP. There was no SBA response in these same 53 serum samples against the homologous NIPH-produced vaccine type strain devoid of class 1 OMP, except 1 sample that had low titer against several heterologous strains. Serum samples from the 14 recipients of NIPH-produced vaccine who had the highest titers against the homologous NIPH-produced vaccine type

strain devoid of class 3 OMP were subsequently tested by SBA against strains expressing B:15:P1.5,2; B:15:P1.19; B:15:P1.7^h,4; B:15:P1.12,13; and B:15:P1.7,10. None of these 14 serum samples showed a response.

Reactogenicity

In general, infants, children, and adults vaccinated with either meningococcal vaccine had more pain, induration and erythema at the site of injection than did control vaccine recipients (TABLE 3). However, more than 95% subjects who reported symptoms stat

Figure. Study Profile for the Randomized Controlled Trial



FI indicates Poly Institute; NIPH, National Institute of Public Health. Control infants and children were inoculated with *Haemophilus influenzae* type B vaccine; control adults were inoculated with aluminum hydroxide adjuvant in solvent.

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that their symptoms did not interfere or only moderately interfered with normal activities. When reported, severe interference with normal activity was more likely to occur among meningococcal vaccine recipients than control vaccine recipients, but interference lasted only 1 to 2 days and was not aggravated by subsequent doses of vaccine. No serious adverse events were attributed to vaccination.

COMMENT

This is, to our knowledge, the first prospective, double-blind, randomized controlled immunogenicity trial comparing proteoliposome OMP vesicle vaccines against serogroup B meningococcal disease among infants and young children. Both vaccines were found to be safe among Chilean infant, children, and adult vaccine recipients; local and systemic reactions were consistent with reactogenicity results from adult trials.^{7A,20}

In the present study, among children and adults who received the FI-

produced and NIPH-produced vaccines, there was a significantly higher proportion of SBA responders against the heterologous Chilean target strain than among those who received the control vaccine. In addition, among children and adults, a third dose of either the FI-produced or NIPH-produced vaccine was associated with a higher proportion of SBA responders than 2 doses. However, among infants, there was no evidence of a significant increase in SBA titers in response to vaccination following 2-dose or 3-dose regimens with either vaccine against the Chilean epidemic strain. These data suggest that neither vaccine would confer sufficient protection during a heterologous epidemic.

Children and adult recipients of the FI-produced and NIPH-produced vaccines demonstrated SBA cross-reactivity to their respective heterologous vaccine strains. Surface components responsible for this heterologous bactericidal activity have not been identified but may include shared lipooli-

gosaccharide immunotype (1,3,7,9), Opa and Ope proteins, or other age-dependent bactericidal epitopes.^{21,24,25}

Infant, children, and adult recipients of the FI-produced and NIPH-produced vaccines showed higher SBA titers against their respective homologous vaccine type strains than against heterologous target strains. Enhanced immunogenicity against homologous vaccine type strains is consistent with findings comparing SBA immunogenicity elicited by the FI-produced and NIPH-produced vaccines among 15- to 20-year-old subjects in Iceland.²⁶ However, because infants receiving both FI-produced and NIPH-produced vaccine in this study showed exceedingly low response rates to the heterologous Chilean epidemic strain, we were surprised to find that infant recipients of the FI-produced and NIPH-produced vaccines demonstrated such high response rates against their respective homologous vaccine type strains.

Of note, the magnitude of homologous SBA response was similar across all

Table 2. Percentage of SBA Responders by Target Strain, Number of Vaccine Doses Received, Vaccine Group, and Age Group*

No. of Vaccine Doses Received†	SBA Responders (Control Responders), %‡					
	FI-Produced Vaccine Group			NIPH-Produced Vaccine Group		
	Infants	Children	Adults	Infants	Children	Adults
	CH1998					
	n = 82 (n = 82)	n = 49 (n = 47)	n = 82 (n = 82)	n = 53 (n = 54)	n = 55 (n = 57)	n = 51 (n = 52)
2	2 (2)	14 (29)	27 (33)	8 (15)	22 (39)	48 (92)
3	10 (12)	31 (63)	37 (45)	12 (23)	35 (61)	60 (92)
	CL1998					
	n = 55 (n = 55)	n = 49 (n = 55)	n = 55 (n = 55)	n = 55 (n = 55)	n = 51 (n = 55)	n = 48 (n = 55)
2	5 (9)	35 (71)	53 (96)	2 (4)	12 (24)	44 (92)
3	50 (91)	75 (92)	57 (95)	2 (4)	24 (47)	45 (94)
	44/76-EL					
	n = 51 (n = 52)	n = 48 (n = 55)	n = 53 (n = 53)	n = 50 (n = 52)	n = 51 (n = 53)	n = 50 (n = 53)
2	15 (14)	22 (46)	42 (79)	35 (14)	75 (71)	55 (49)
3	31 (29)	41 (85)	55 (100)	35 (29)	55 (53)	55 (52)
	44/76-EL Without Class 3 OMP, n = 53					
3	...	...	...	...	...	...
	44/76-EL Without Class 1 OMP, n = 53					
3	...	...	...	...	...	...

*SBA indicates serum bactericidal activity; FI, Frilly Institute; NIPH, National Institute of Public Health; CH1998, 1998 Chilean epidemic strain; CL1998, FI-produced vaccine type strain; 44/76-EL, NIPH-produced vaccine type strain; OMP, outer-membrane protein; and ellipses, data not applicable.

†All vaccines were administered as a 3-dose regimen, with each dose given 2 months apart; blood samples were obtained prior to dose 1 and approximately 8 weeks following the second dose and 4 to 6 weeks following the third dose of vaccine.

‡A responder was defined as a person with a 4-fold or greater rise in antibody titer compared with pre-vaccination titer. Infant and child controls received Neisseria meningitidis polysaccharide vaccine; adult controls received aluminum hydroxide adjuvant dissolved in solvent.

§P < .05 vs control using the Yates corrected χ^2 test.

¶P < .001 vs control using the Yates corrected χ^2 test.

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3 age groups. The prevaccination geometric mean titers were essentially 2 or less across all 3 age groups in both homologous assays. Among FI-produced vaccine recipients, 67.9% of infants, 67.4% of children, and 67.3% of adults had a geometric mean SBA titer of 1:8 or greater following the third dose of vaccine (2-tailed Fisher exact test, $P > .99$). Similarly, among NIPH-produced vaccine recipients, 96.4% of infants, 100% of children, and 96.0% of adults had a geometric mean SBA titer of 1:8 or greater following the third dose of vac-

cine (2-tailed Fisher exact test, $P = .47$) (data available from the authors on request). Although SBA may be a less sensitive measure of protection than clinical vaccine efficacy, SBA is likely to be an acceptable serologic correlate for estimating the protective potential of OMP serogroup B meningococcal vaccines.^{14,20,23}

In 1989 and 1990, the FI-produced vaccine was used during a homologous serogroup B meningococcal epidemic in São Paulo.⁸ Age-dependent differences in vaccine efficacy among

children aged 3 months to 6 years were observed. Vaccine efficacy was not demonstrated among children younger than 4 years following a 2-dose regimen, suggesting that the FI-produced vaccine did not confer protection against disease caused by the São Paulo strain in young children.⁸ In our study, infant recipients of the FI-produced vaccine had nearly a doubling in their homologous SBA response rate following a third dose of vaccine suggesting that a 3-dose regimen may have had a positive effect on vaccine efficacy among chil-

Table 2. Frequency of Local and Systemic Reactions Among Participants by Study Age Group and Vaccine Group*

Symptoms and Signs	Dose 1 (Control Group), %			Dose 2 (Control Group), %			Dose 3 (Control Group), %		
	FI (FI)	NIPH (NIPH)	FI (NIPH)	FI (FI)	NIPH (NIPH)	FI (NIPH)	FI (FI)	NIPH (NIPH)	FI (NIPH)
Infants									
	n = 62 (n = 62)	n = 62 (n = 62)	n = 62 (n = 62)	n = 61 (n = 62)	n = 61 (n = 62)	n = 61 (n = 62)	n = 60 (n = 61)	n = 60 (n = 61)	n = 60 (n = 62)
Temperature $\geq 38^{\circ}\text{C}$	21 (13)	21 (13)	21 (21)	25 (23)	31 (23)	28 (31)	40 (28)	25 (22)	40 (22)
Vomiting	13 (11)	8 (11)	13 (8)	7 (10)	8 (10)	7 (8)	20 (31)	8 (8)	20 (8)
Poor appetite	18 (11)	18 (11)	18 (15)	20 (18)	22 (18)	20 (22)	23 (31)	24 (31)	23 (24)
Irritability	58 (51)	58 (51)	58 (38)	44 (44)	47 (44)	44 (47)	55 (38)	48 (38)	55 (48)
Pain	25 (13)	15 (13)	25 (15)	18 (11)	22 (11)	18 (22)	23 (13)	7 (13)	23 (7)
Induration	53 (11)	45 (11)	53 (45)	33 (11)	34 (11)	33 (34)	38 (31)	41 (31)	38 (41)
Erythema	40 (13)	34 (13)	40 (34)	33 (13)	27 (15)	33 (27)	30 (10)	32 (10)	30 (32)
Children									
	n = 60 (n = 62)	n = 61 (n = 62)	n = 60 (n = 61)	n = 58 (n = 62)	n = 58 (n = 62)	n = 58 (n = 62)	n = 48 (n = 62)	n = 58 (n = 62)	n = 48 (n = 62)
Temperature $\geq 38^{\circ}\text{C}$	23 (18)	20 (18)	23 (23)	12 (23)	19 (23)	12 (18)	10 (27)	13 (27)	10 (13)
Vomiting	5 (3)	10 (3)	5 (10)	2 (7)	0 (7)	2 (3)	8 (3)	9 (3)	8 (8)
Poor appetite	28 (18)	21 (18)	28 (21)	19 (7)	10 (7)	19 (10)	12 (20)	14 (23)	12 (14)
Irritability	45 (38)	25 (38)	45 (38)	48 (23)	28 (23)	48 (23)	35 (28)	32 (28)	35 (32)
Pain	45 (27)	20 (27)	45 (23)	60 (42)	47 (42)	60 (47)	58 (31)	48 (31)	55 (48)
Induration	48 (21)	35 (21)	48 (35)	40 (22)	43 (22)	40 (43)	51 (15)	21 (15)	51 (21)
Erythema	40 (18)	33 (18)	40 (33)	38 (13)	38 (13)	38 (38)	36 (10)	34 (10)	36 (34)
Adults									
	n = 58 (n = 57)	n = 57 (n = 57)	n = 58 (n = 57)	n = 58 (n = 58)	n = 53 (n = 58)	n = 55 (n = 53)	n = 53 (n = 54)	n = 48 (n = 54)	n = 53 (n = 48)
Temperature $\geq 38^{\circ}\text{C}$	8 (4)	2 (4)	8 (2)	5 (4)	5 (4)	5 (5)	4 (8)	6 (8)	4 (8)
Headache	40 (37)	51 (37)	40 (31)	38 (31)	40 (31)	38 (40)	48 (28)	38 (28)	48 (38)
Nausea	8 (11)	11 (11)	8 (11)	7 (7)	13 (7)	7 (13)	8 (15)	4 (13)	8 (4)
Myalgia	38 (28)	38 (28)	38 (33)	38 (31)	40 (31)	38 (40)	40 (17)	38 (17)	40 (38)
Pain	51 (44)	51 (44)	51 (31)	78 (38)	58 (38)	78 (38)	51 (31)	55 (31)	51 (55)
Induration	48 (11)	32 (11)	48 (38)	25 (14)	34 (18)	25 (34)	34 (7)	35 (7)	34 (35)
Erythema	22 (3)	25 (3)	22 (35)	37 (3)	25 (7)	27 (25)	25 (3)	19 (3)	23 (19)

*FI indicates Finlay Institute; NIPH, National Institute of Public Health. Infant and child age group controls received *Haemophilus influenzae* type b conjugate vaccine (FI); adult controls received aluminum hydroxide adjuvant diphtheria in islet (AIDF).

† $P < .05$ vs control using the Yates corrected χ^2 test.

‡ $P < .001$ vs control using the Yates corrected χ^2 test.

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dren vaccinated in São Paulo. Age-dependent vaccine efficacy was also shown for a serogroup B meningococcal vaccine produced by the Walter Reed Army Institute of Research, Washington, DC, and evaluated in Iquique in 1992.¹⁰ This OMP vaccine based on a Chilean outbreak strain was not efficacious among children younger than 5 years. However, the Walter Reed Army Institute vaccine trial was administered as a 2-dose regimen, the vaccine did not present its OMPs as proteoliposome vesicles, and the vaccine contained only 0.1% lipopolysaccharide,¹⁰ factors that may have contributed to vaccine failure in young children. Of note, in both the São Paulo and Iquique vaccine efficacy studies, poor SBA responses were associated with vaccine failure among children younger than 4 and 5 years, respectively, whereas higher SBA responses were associated with protection.^{10,20}

In the current study, infant recipients of the NIPH-produced vaccine demonstrated a nearly uniform response (98%) against the isogenic, class 3 OMP-deficient, NIPH-produced vaccine type strain, strongly suggesting that class 1 OMP was the immunodominant antigen responsible for the elicited bactericidal immunogenicity. This finding, in conjunction with established serogroup B OMP vaccine efficacy estimates for older children and adults,⁷⁻¹⁰ has profound implications for serogroup B vaccine development in response to epidemics. Although serogroup B epidemics are seasonal, they generally persist in the affected population for several years to decades.^{3,17-19,43} This persistence, however unfortunate, provides OMP vaccine producers ample time to develop and rapidly produce an epidemic strain-specific vaccine following the careful selection of a vaccine type strain. This strategy is being actively pursued in New Zealand in response to a highly clonal epidemic.

Class 1 OMP is conventionally classified by using serosubtyping methods that characterize the *porA* OMP variable region epitopes with monoclonal antibodies.⁴⁴ DNA sequence data sug-

gest that the current panel of serosubtype-defining monoclonal antibodies underestimates *porA* variable region variability.^{33,45-48} Because minor changes in the variable region can dramatically alter immune recognition,³⁰ DNA sequencing of a representative sample of epidemic serogroup B strains is imperative for the selection of an epidemic vaccine type strain. Although a recent study found the *porA* gene to be stable for up to 30 weeks in persons with prolonged nasopharyngeal carriage,³³ some evidence suggests that the *porA* gene may not be stable over a period of decades.⁴⁹ Therefore, DNA sequencing of a representative sample of epidemic strains throughout the epidemic period may also be important.

Endemic meningococcal disease occurs worldwide.³ Serogroup B accounts for nearly half of the 2600 endemic cases that occur annually in the United States³¹ and two thirds of the 1800 endemic cases of meningococcal disease reported in England and Wales.⁵² Laboratory-based surveillance for *N meningitidis* is a reliable method of identifying and monitoring class 1 OMP antigens (and the stability of the *porA* gene) of circulating strains over time.³¹⁻³³ In the United States, a limited number of class 1 proteins represent more than three fourths of class 1 proteins expressed by endemic serogroup B isolates, suggesting that an efficacious, multivalent class 1 OMP vaccine could prevent endemic meningococcal disease. However, DNA sequencing of these strains will be necessary to evaluate *porA* variable region variability.⁵⁴ A hexavalent, serogroup B class 1 OMP meningococcal vaccine has been developed^{28,29} and is being tested in immunogenicity trials in the United Kingdom.⁵⁵ Further research and development of multivalent serogroup B meningococcal vaccines based on highly endemic and epidemic strain-specific class 1 OMP antigens is warranted.

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Immunogenicity studies with a genetically engineered hexavalent PorA and a wild-type meningococcal group B outer membrane vesicle vaccine in infant cynomolgus monkeys

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Abstract

The immunogenicity of two meningococcal outer membrane vesicle (OMV) vaccines, namely the Norwegian wild-type OMV vaccine and the Dutch hexavalent PorA OMV vaccine, were examined in infant cynomolgus monkeys. For the first time, a wild-type- and a recombinant OMV vaccine were compared. Furthermore, the induction of memory and the persistence of circulating antibodies were measured. The Norwegian vaccine contained all four classes of major outer membrane proteins (OMP) and wild-type L3/L8 lipopolysaccharide (LPS). The Dutch vaccine consisted for 90% of class 1 OMPs, had low expression of class 4 and 5 OMP, and GalE LPS. Three infant monkeys were immunized with a human dose at the age of 1.5, 2.5 and 4.5 months. Two monkeys of each group received a fourth dose at the age of 11 months. In ELISA, both OMV vaccines were immunogenic and induced booster responses, particularly after the fourth immunisation. The Norwegian vaccine mostly induced sero-subtype P1.7,16 specific serum bactericidal antibodies (SBA), although some other SBA were induced as well. The antibody responses against P1.7,16, induced by the Norwegian vaccine, were generally higher than for the Dutch vaccine. However, the Dutch vaccine induced PorA specific SBA against all six sero-subtypes included in the vaccine showing differences in the magnitude of SBA responses to the various PorAs. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Meningococcal vaccine; PorA; Monkey

1. Introduction

Bacterial meningitis caused by *Neisseria meningitidis* serogroup B strains is a life-threatening disease. Especially young infants (<18 months) are at highest risk. The group B capsular polysaccharide (CPS) is not immunogenic in man, most likely due to structural

similarities with host components [1]. Improvement of the immunogenicity of serogroup B CPS by chemical modification and/or conjugation of the B polysaccharide resulted in alteration of the antigenic properties. Some success has been obtained and this approach is being continued [2,3].

Two important observations have led to another vaccine approach, which is based upon the use of outer membrane components, such as outer membrane proteins (OMP) and lipopolysaccharides (LPS). Firstly, in the case of serogroup B infections, serum bactericidal antibodies (SBA) are directed against non-capsular surface antigens [4,5] and secondly, the presence of SBA is correlated with protection against

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Table 1
Vaccine composition of a human dose (0.5 ml) of the Dutch and the Norwegian OMV vaccine^a

Contents	Dutch OMV vaccine lot B9281	Norwegian OMV vaccine lot MV191A
Total protein (µg)	100	25
High mol. wt. (%) (kD)	2 (80)	5 (70+80)
PorA (%)	89–90	33
Subtypes	P1.7,16;1,2;19,15; P1.5 ² ;10;12,13;7 ³ ,4	P1.7,16
PorB serotype 15 (%)	absent	38
Rmp (%)	7	12
Opa/Opc (%)	3	15
LPS (µg), immunotype	10, GalE	1.75 wild-type (L3, L8)
Phospholipid (µg)	nd	2
B CPS (µg)	absent	< 0.085
DOC (µg)	11	4
AlPO ₄ (mg)	1.5	–
Al(OH) ₃ (mg)	–	1.67
Sucrose (mg)	10	9
Thiomersal (mg)	0.05	0.05

^a PorA: class 1 outer membrane protein (OMP); PorB: class 2/3 OMP; Rmp: class 4 OMP (reduction modifiable protein); DOC: deoxycholate; LPS: lipopolysaccharide; B CPS: group B capsular polysaccharide; Opa/Opc: class 5 OMPs; nd: not determined.

meningococcal diseases [6]. Clinical studies with meningococcal outer membrane vesicle (OMV) vaccines, based on single epidemic strains, have been shown to induce mostly sero-subtype-specific, but also some cross-reactive bactericidal responses [7,8]. The immune responses, as induced by OMV-based vaccines, were found to correlate with protection against disease [9,10]. Most of the antibodies reacted with the PorA porin protein, one of the major outer membrane proteins of meningococci. This is in accordance with previous animal studies, which showed PorA as a potent inducer of SBA [11]. The phase-variable Opc protein has also been found to induce SBA after vaccination in man [12].

In the late 1980s, an outer membrane vesicle (OMV) vaccine was developed in Norway based on wild-type case-isolate H44/76, B:15:P1.7,16, a representative for an ongoing epidemic [13,14]. An efficacy study was carried out in teenagers and young adults receiving two immunisations 6 weeks apart, which showed a protection rate of approximately 57%, as measured during a 2.5-year follow-up period [7,15]. The SBA was mainly directed against the PorA and Opc proteins. Booster responses were found when a third immunisation was administered after 4–5 years and after 11 months [7,8]. The immunogenicity of this vaccine was also studied in infants and children in Chile [16].

In the Netherlands, a hexavalent PorA-based OMV vaccine was developed, which contains the six predominant PorA-subtypes as isolated in many countries [17,18]. Two trivalent PorA vaccine strains based on wild-type strain H44/76 were constructed by recombinant DNA techniques. In addition, these triple PorA strains are deficient in the expression of some other surface antigens such as PorB, wild-type lipopolysac-

charide (LPS) and group B capsular polysaccharide (CPS) [17,18]. Clinical studies were carried out in adult volunteers, receiving from one to three doses of the vaccine, respectively [19–21]. A strong bactericidal immune response directed against PorA was induced, although no booster responses were observed. Recently, the immunogenicity and reactogenicity of this vaccine were tested in infants in UK [22]. The hexavalent PorA OMV vaccine was shown to be safe and immunogenic although multiple doses of vaccine were required to induce a significant SBA response and there were differences in the magnitudes of SBA responses to different PorA's [22].

In the present study the immunogenicity of the Dutch hexavalent PorA and the Norwegian OMV vaccines were compared after three to four immunisations administered to infant cynomolgus monkeys starting at the age of 6 weeks. Particular attention was given to the specificity of the induced SBA by using well-characterised target strains.

2. Materials and methods

2.1. Outer membrane vesicle (OMV) vaccines

Two OMV vaccines, namely the Dutch hexavalent PorA OMV vaccine and the Norwegian wild-type OMV vaccine, were used in this study (see Table 1). The Dutch OMV vaccine is a genetically engineered hexavalent PorA OMV vaccine (lot B 9281) that contains class 1 OMP of six meningococcal subtypes [18]. The subtypes are expressed on two trivalent vesicles P1.7,16; P1.19,15; P1.5,2 and P1.5c,10; P1.12,13; P1.7³,4. Production, characterisation and control of

the hexavalent PorA OMV vaccine were described by Claassen et al. [17]. The safety and immunogenicity of the vaccine were tested in adults [19] and infants [22].

The Norwegian OMV vaccine (lot MV1 91A) contained OMVs from a clinical isolate, H44/76 (B:15:P1.7,16), of a fatal case of meningitis and septicaemia in 1976 [14]. Production, characterisation and control of the wild-type OMV vaccine was described by Holst Fredriksen et al. [13]. The safety and immunogenicity of the Norwegian OMV vaccine were described in teenagers and young adults [15,16] and children and infants [16].

2.2. Monkeys and immunisations

Infant cynomolgus monkeys (*Macaca fascicularis*) were bred at the RIVM and kept by their mothers in small groups according to standard regulations. Because of a limited availability of monkeys, only three animals could be vaccinated with each OMV vaccine. At the age of 1.5 month (week 0), two groups of infant monkeys ($n=3$) were immunised intramuscularly with one human dose (0.5 ml) of the Dutch (100 µg protein per dose; infant monkeys K136f2, K137f2 and K138f2) or the Norwegian (25 µg protein per dose; infant monkeys H9, H10 and H11) OMV vaccine. Booster immunisations with the homologous vaccine were administered at the age of 2.5 and 4.5 month, after 4 and 12 weeks, respectively. Monkeys were bled prior to each immunisation and subsequently every two weeks up to week 16 after the first dose. After week 16, two infant monkeys of each group were bled subsequently every two months till week 39–41 (age 11 months) and a fourth booster immunisation was administered. Blood samples were drawn prior to the fourth immunisation and subsequently every two weeks for a period of 4–8 weeks postimmunisation. Sera were stored at -20°C . Both OMV vaccines were well tolerated by the animals. No serious adverse events occurred. No abnormal behaviour was observed during the experiments.

2.3. ELISA

OMV preparations were derived from vaccine strains H44/76 (B:15:P1.7,16), PL16215 (–:P1.7,16,5,2,19,15) and PL10124 (–:P1.5^o,10,12,13,7^o,4). Heterologous OMVs were derived from strains PL9146 (–:P1.9,14,6) expressing three PorAs not present in the vaccines, and from a wild-type reference strain 2996 (B:2b:P1.5,2). All strains have been described previously [14,18,19,24]. The OMV preparations were produced according to the production method of the Dutch hexavalent PorA OMV vesicle vaccine [17]. Immune responses of the infant monkeys were measured by enzyme-linked

Table 2

Phenotypic characteristics of meningococcal strains used as target in the bactericidal assay

Strain ^a	Phenotype	Origin
1 H44/76 P1.7,16	B:15:P1.7,16	original H44/76 case-isolate
2 TR52	B:15:P1.5,2	isogenic H44/76
3 TR15	B:15:P1.19,15	isogenic H44/76
4 TR10	B:15:P1.5 ^o ,10	isogenic H44/76
5 TR1213	B:15:P1.12,13	isogenic H44/76
6 TR4	B:15:P1.7 ^o ,4	isogenic H44/76
7 H15	B:15:P1.–	isogenic H44/76
8 2996	B:2b:P1.5,2	wild-type patient isolate

^a All strains have been described previously [14,19,24].

immuno-sorbent assay (ELISA) as described previously [20]. Peroxidase-labelled rabbit anti-human IgG (DAKO, Denmark) diluted 1:5000 that cross-reacts with cynomolgus monkeys IgG was used as conjugate [23]. Titres were expressed as reciprocal dilutions giving an absorbance of 1.0 at 450 nm.

2.4. Bactericidal assay

The phenotypic characteristics of the target strains used in the bactericidal assay are shown in Table 2. Strains H44/76 (B:15:P1.7,16) and the six isogenic PorA strains (TR52, TR15, TR1213, TR4 and TR10) were used to characterise the SBA against individual PorAs. Strain H15, a PorA-deficient (B:15:P1.–) isogenic variant was used to determine if the induced SBA was PorA specific. In addition a wild-type reference strain, 2996 (B:2b:P1.5,2) was used. All strains have been described previously [14,19,24].

The bactericidal assay was carried out as described previously [19]. Briefly, all serum samples of the monkeys were heat-inactivated for 30 min at 56°C and tested in twofold dilution series starting at 1/4 against the selected strains. Bactericidal sero-subtype specific MAbs and a human serum with known bactericidal activity were included in each day's experiment as internal control. A human serum with no bactericidal activity against the target strains was used as an external complement source at a final concentration of 10%. The bactericidal titre is reported as the highest reciprocal serum dilution showing killing ($>90\%$) of the bacteria (by visual inspection).

3. Results

3.1. Antibody responses induced by the OMV vaccines measured by ELISA

3.1.1. Dutch hexavalent PorA OMV vaccine

Sera from the individual infant monkeys K136f2,

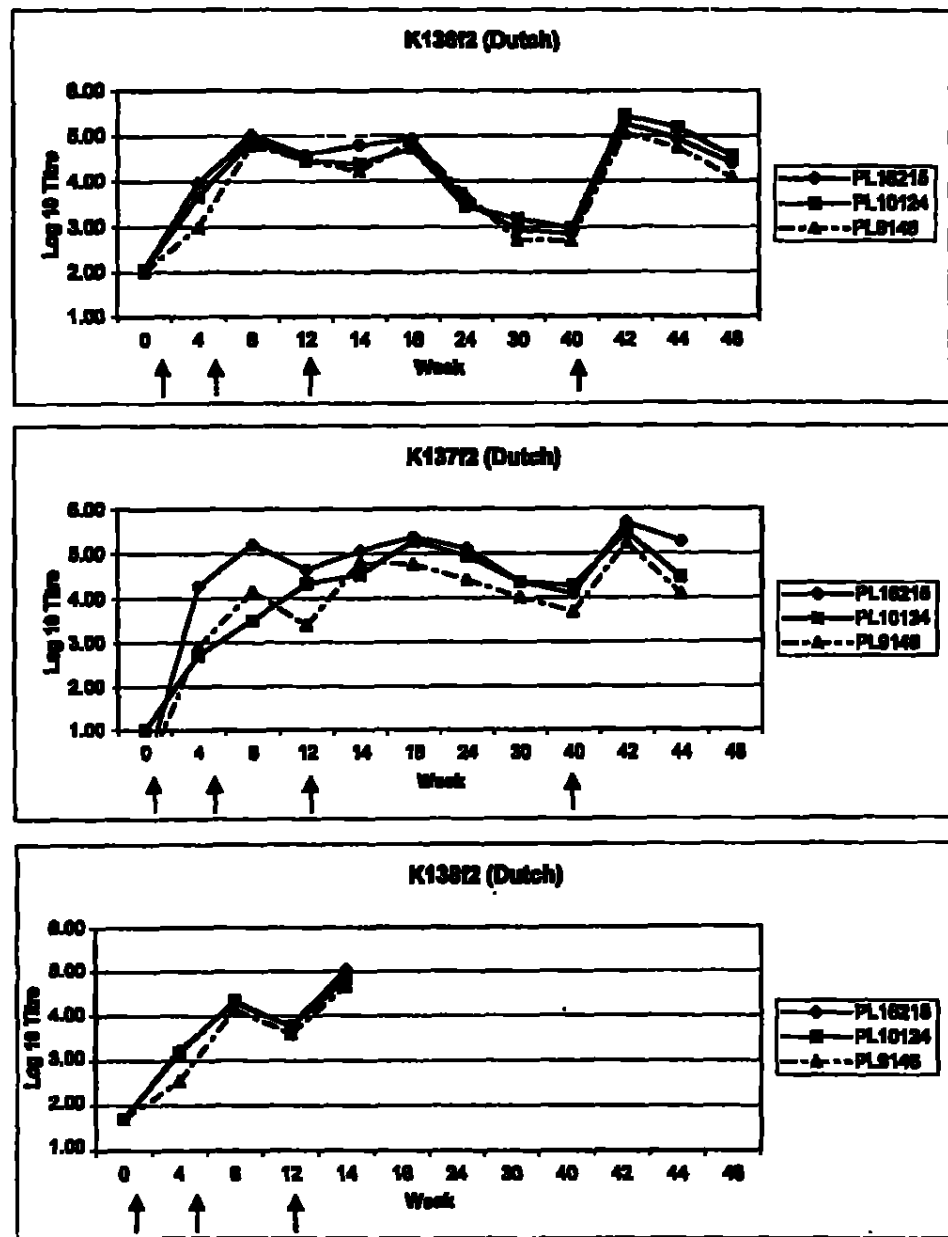


Fig. 1. IgG antibody responses of individual infant monkeys immunised with the Dutch hexavalent PorA OMV vaccine determined in ELISA. The results are shown with OMV preparations of vaccine strains PL16215 (---P1.7,16,5,2,19,15) and PL10124 (---P1.5*,10,12,13,7*,4) and the heterologous strain PL9146 (---P1.9,14,6) as antigen. Titres were expressed as reciprocal dilutions giving an absorbance of 1.0 at 450 nm. Arrows indicate times of immunisation.

K137f2 and K138f2 were tested against the homologous trivalent vaccine PL16215 OMVs and PL10124 OMVs and against the heterologous PL9146 OMVs. The total IgG antibody responses induced with the Dutch OMV vaccine after consecutive immunisations are shown in Fig. 1.

IgG antibodies to PL16215 OMVs could already be

detected four weeks after the first dose given week 0 in all three animals. Lower IgG responses were found to PL10124 OMVs and the heterologous PL9146 OMVs. Four weeks after the second dose (week 8), a marked rise in IgG antibody titre was observed against PL16215 OMVs for monkeys K136f2 and K137f2. K136f2 showed also an increase in antibodies against

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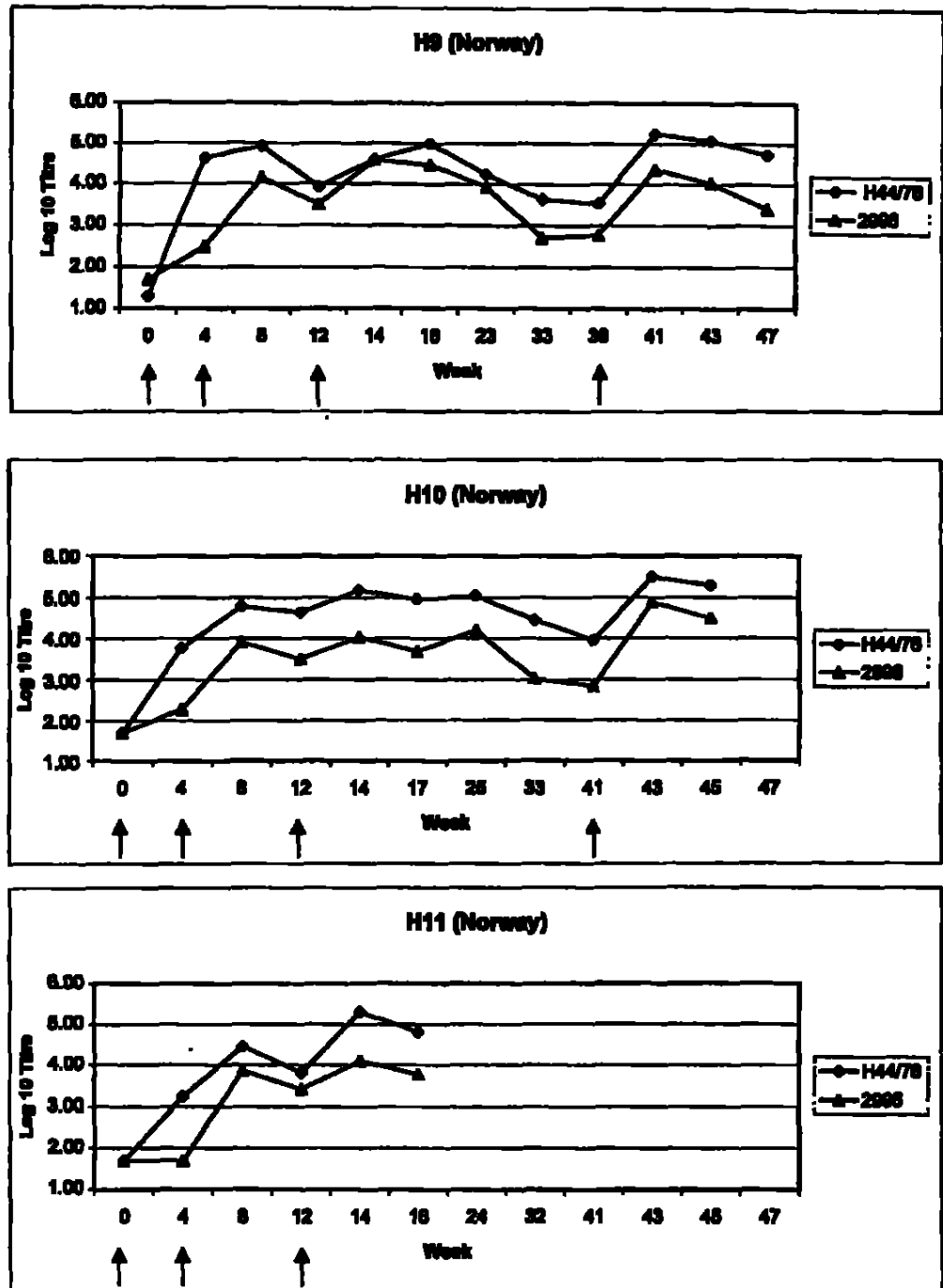


Fig. 2. IgG antibody responses of individual infant monkeys immunised with the Norwegian CIMV vaccine determined in ELISA. The results are shown with CIMV preparations of vaccine strain H44/76 (R15:P1.7J6) and the heterologous strain 2996 (R2b:P1.5J2) as antigen. Titres were expressed as reciprocal dilutions giving an absorbance of 1.0 at 450 nm. Arrows indicate times of immunisation.

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PL10124 OMVs and against PL9146 OMVs. A lower increase in IgG antibody titre was observed in monkey K138f2 after the second dose. Eight weeks after the second dose (week 12), a decline in antibody titres was observed in all three monkeys. The third immunisation induced high IgG responses in all three monkeys against the two OMVs of the vaccine and against heterologous PL9146 OMVs as measured 2–4 weeks later (week 14–16). Compared to the first two doses, the highest responses were measured 4 weeks after the third immunisation (week 16) for monkey K137f2. The antibody response was directed against OMVs of both vaccine strains, the responses were higher and persisted longer (12 weeks) than the responses after the second immunisation. The response of K136f2 after the third dose was similar to that after the second dose. Twelve to 18 weeks after the third dose, again a decline in antibody titres (week 24/30) was observed, especially in monkey K136f2.

A fourth dose was given to monkeys K136f2 and K137f2 at week 40 when the monkeys were 11 months old (Fig. 1). This dose induced a strong IgG response against PL16215 OMVs and PL10124 OMVs in both monkeys despite the different levels of specific antibodies still present. The IgG-titres against the vaccine vesicles were markedly higher compared to the titres after the second and third dose.

3.1.2. Norwegian OMV vaccine

Sera from the individual infant monkeys H9, H10 and H11 immunised with the Norwegian OMV vaccine were tested homologous vaccine OMVs from wild-type strain H44/76 (B:15:P1.7,16) and heterologous OMVs from wild-type strain 2996, B:2b:P1.5,2. Total IgG antibody responses induced with the Norwegian OMV vaccine after consecutive immunisations are shown in Fig. 2. Anti-H44/76 OMVs IgG antibodies were detected in sera from all three monkeys four weeks after the first dose. The antibody titres of monkey H9 and H10 were higher compared to those in H11. Four weeks after the second dose, an increase of specific IgG antibodies was detected in sera from all monkeys, especially H10, against the H44/76 OMVs. Lower IgG titres were found against 2996 OMVs. The titres decreased in the following weeks especially in monkey H9 and H10. These monkeys responded two weeks after the third immunisation (week 14) with a marked increase in IgG antibody titre against the H44/76 vaccine OMVs. H10 only showed a small increase in IgG titre to H44/76 OMVs. At the same time, the IgG titre to 2996 OMVs increased in H9 and H11. For both monkeys, IgG titres against H44/76 OMVs persisted till 19 weeks after the third dose (week 33). The IgG titre of monkey H11 declined already four weeks after the third dose. A fourth dose was given to monkeys

H9 and H10 at the age of 11 months (seven months after the third dose, week 39–41). Two weeks after the fourth dose a strong IgG response was detected against the H44/76 OMVs in both monkeys. This response was higher compared to those after the second and third dose.

3.2. Bactericidal antibody responses induced by the OMV vaccines

3.2.1. Dutch hexavalent PorA OMV vaccine

The results for the monkeys immunised with the Dutch OMV vaccine are shown in Table 3. The induced SBA were specific for PorA, as no SBA were found with strain H15 at any time point. A strong bactericidal immune response was already induced 4 weeks after the first immunisation against the majority (5 to 6) of the isogenic strains expressing the six PorAs in the vaccine in all monkeys. The Dutch OMV vaccine induced the highest titres specific for PorAs P1.5,2 (present in PL16215) and P1.5^o,10 (present in PL10124) in all three monkeys at week 4. Two monkeys (K136f2, K137f2) also showed strong SBA specific for PorAs P1.7,16 (present in PL16215) and P1.12,13 (present in PL10124). An intermediate response was found for monkey K136f2 against PorAs P1.19,15 and P1.7^b,4. This monkey responded to all six strains expressing the PorAs represented in the Dutch OMV vaccine. Monkey K137f2 showed an increase in SBA specific for PorA P1.19,15, but no activity was found for PorA P1.7^b,4. Monkey K138f2 showed, besides the strong responses specific for the strains expressing PorAs P1.5,2 and P1.5^o,10, also intermediate responses specific for the strains expressing PorAs P1.19,15, P1.12,13 and P1.7^b,4. The Dutch OMV vaccine induced in all three monkeys PorA-specific SBA for wild-type strain 2996, which expresses the same PorA P1.5,2 as in the Dutch vaccine. The titres against 2996, however, were lower compared to titres against isogenic strain H44/76 P1.5,2 (B:15:P1.5,2).

The observed immunodominance of PorA sero-subtypes P1.5,2 and P1.5^o,10 compared to the other four PorAs remained after the second and third dose, which was also reflected in the SBA against wild-type strain 2996 (B:2b:P1.5,2). SBA against the other PorAs declined compared to responses after the first dose, but some activity was observed for monkeys K136f2 and K137f2 up to 28 weeks after the third immunisation. These remaining SBA were at least 4-fold higher compared to prevaccination levels and was directed against two to four of the six vaccine antigens.

The fourth dose induced strong bactericidal booster responses in monkeys K136f2 and K137f2 directed against three to four of the PorA target strains. Also

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Table 3

Serum bactericidal antibody responses in infant monkeys after multiple immunisations with 100 µg of the Dutch Homovalent PorA outer membrane vesicle vaccine^a

Week	Target strains							
	H44/76 B:15:P1.7,16	TR52 B:15:P1.5,2	TR15 B:15:P1.19,15	TR10 B:15:P1.5a,10	TR1213 B:15:P1.12,13	TR4 B:15:P1.7h,4	H15 B:15-	2996 B:2b:P1.5,2
K136/2								
→ 0	< 4	< 4	< 4	< 4	< 4	< 4	< 4	< 4
→ 4	256	512	8	512	64	16	< 4	128
8	128	512	< 4	64	16	< 4	< 4	32
→ 12	128	128	< 4	128	16	< 4	< 4	8
16	32	256	< 4	1024	64	< 4	< 4	128
24	< 4	8	< 4	128	8	< 4	< 4	8
32	< 4	4	< 4	64	< 4	< 4	< 4	< 4
→ 40	< 4	16	< 4	32	< 4	< 4	< 4	< 4
42	512	1024	< 4	nd	256	16	< 4	nd
44	256	512	< 4	nd	64	< 4	< 4	128
48	16	64	< 4	nd	16	< 4	< 4	8
K137/2								
→ 0	< 4	< 4	< 4	< 4	< 4	< 4	< 4	< 4
→ 4	256	512	8	512	64	< 4	< 4	32
8	256	256	16	64	16	< 4	< 4	8
→ 12	64	512	16	128	32	8	< 4	16
16	32	1024	16	256	128	16	< 4	512
24	32	256	< 4	32	32	8	< 4	64
32	16	128	< 4	16	16	4	< 4	32
→ 40	8	64	< 4	8	8	< 4	< 4	32
42	512	1024	8	> 2048	256	8	< 4	1024
44	256	256	4	512	128	8	< 4	256
K138/2								
→ 0	< 4	< 4	< 4	< 4	< 4	< 4	< 4	< 4
→ 4	< 4	128	32	128	16	16	< 4	32
8	< 4	128	< 4	128	4	< 4	< 4	16
→ 12	4	32	< 4	128	4	< 4	< 4	< 4
16	128	128	< 4	512	16	< 4	< 4	16

^a Bactericidal titres are reported as reciprocal serum dilutions showing >90% killing; nd: not determined; →: immunisation.

after the fourth dose an immunodominance was found for PorAs P1.5,2 and P1.5^a,10 followed by PorAs P1.7,16 and P1.12,13 (Table 3, week 42). This was reflected in strong SBA against wild-type strain 2996. Overall, a clear difference in magnitude was observed with respect to the induced bactericidal antibodies against the six sero-subtypes in the Dutch OMV vaccine; PorAs P1.19,15 and P1.7^h,4 were the least immunogenic.

3.2.2. Norwegian OMV vaccine

Four weeks after the first dose, strong SBA was detected in sera from monkeys H9 and H10 against vaccine strain H44/76(B:15:P1.7,16) (Table 4). The SBA of monkey H11 was low compared to the other

two monkeys. Monkeys H9 showed no activity with isogenic strain H15 (PorA-deficient) at any time point. Monkeys H10 and H11 showed intermediate titres against this strain. None of the animals showed any SBA to wild-type strain 2996 (B:2b:P1.5,2), but intermediate titres against TR52 (B:15:P1.5,2) were observed. The bactericidal titres of sera from monkeys H9 and H10 against vaccine strain H44/76 P1.7,16 showed no further increase 4 weeks after the second dose. However, for monkey H11, the second dose induced a high SBA response against the vaccine strain. At week 12, when the third dose was given, the SBA responses of monkeys H9 and H10 had decreased to low levels, whereas monkey H11 still showed a high titre against the vaccine strain. Four weeks after the

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Table 4

Serum bactericidal antibody responses in infant monkeys after multiple immunisations with 25 µg of the Norwegian H44/76 derived outer membrane vesicle vaccines^a

Week	Target strains			
	H44/76 B:15:P1.7,16	TR52 B:15:P1.5,2	H15 B:15-	2996 B:2b:P1.5,2
H9				
→ 0	< 4	< 4	< 4	< 4
→ 4	128	16	< 4	< 4
8	128	16	< 4	< 4
→ 12	4	< 4	< 4	< 4
16	1024	4	< 4	< 4
23	64	< 4	< 4	< 4
33	32	< 4	< 4	< 4
→ 39	16	< 4	< 4	< 4
41	1024	32	< 4	< 4
43	512	< 4	< 4	< 4
47	128	< 4	< 4	< 4
H10				
→ 0	< 4	< 4	< 4	< 4
→ 4	512	16	32	< 4
8	256	8	16	< 4
→ 12	4	4	16	< 4
17	128	< 4	4	< 4
25	32	< 4	< 4	< 4
→ 41	4	< 4	< 4	< 4
43	512	< 4	< 4	< 4
45	128	< 4	< 4	< 4
H11				
→ 0	< 4	< 4	< 4	< 4
→ 4	4	8	8	< 4
8	256	32	8	< 4
→ 12	128	32	8	< 4
16	> 2048	32	8	< 4

^a Bactericidal titres are reported as reciprocal serum dilutions showing > 90% killing; →: immunisation.

third dose (week 16), all three monkeys showed a good booster response against the vaccine strain H44/76 P1.7,16. Although monkey H11 still showed a strong SBA activity against vaccine strain H44/76 P1.7,16 of 1/128 at week 12, a strong booster response was induced by the third immunisation as measured at week 16 (> 1/2048).

The SBA declined gradually after the third dose, although remaining activity was observed against vaccine strain H44/76 P1.7,16 for monkeys H9 (week 23,33,39) and H10 (week 25 and 41).

The fourth dose induced good booster responses in monkeys H9 and H10 after two weeks against vaccine strain H44/76 P1.7,16. All observed responses were PorA-dependent, as no or low SBA were detected against the other strains.

4. Discussion

For the first time, two types of meningococcal OMV vaccines, namely a conventional wild-type OMV vaccine versus a recombinant OMV vaccine, were compared for their immunogenicity in infant cynomolgus monkeys. Both vaccines shared the same meningococcal background, since they were both derived from parental strain H44/76. However, the OMV vaccines vary with respect to the amount of total protein, the sero-subtypes of proteins and LPS-immunotypes present within the OMVs and the adjuvant used.

In general, both vaccines induced sero-subtype specific immune responses in ELISA and bactericidal assay. Both OMV vaccines already induced OMV specific IgG-responses after the first dose similar to human studies [7,8,19,21,22]. The antibody levels decreased after the first two injections, however booster responses were noted independent of the antibody level present. This indicates that immunological memory was induced by the first immunisations. The effect of a booster dose was also observed in adults [7,16] and in infants in Chile [16] using the Norwegian OMV vaccine.

Serum bactericidal activity has become the primary serologic assay used to assess protective immunity stimulated by serogroup B meningococcal vaccine candidates [7-9,12,16,19-22]. However, the lowest level titre of SBA consistent with protection against invasive serogroup B meningococcal disease has not been determined. For the Dutch OMV vaccine, the bactericidal activity was directed against most of the PorAs present in the vesicle vaccine and the strongest booster responses were found against PorAs P1.5,2 and P1.5⁹,10. The immunodominance for some of the PorAs, particularly P1.5,2 and P1.5⁹,10, within each trivalent vaccine vesicle confirmed previous findings with respect to the bactericidal activity induced by the Dutch OMV vaccine in adults [21] and infants [22]. The bactericidal antibodies against sero-subtypes P1.7,16 and P1.12,13 were generally high as well. The responses against P1.7⁸,4 and P1.19,15 were lower, suggesting that some PorA are less immunogenic compared to other PorAs or that three PorAs presented by one vesicle might be a suboptimal formulation. Studies in mice indicate that the difference in magnitude of response to various PorAs is not due to the presentation of three PorAs on one vesicle but due to the characteristics of the PorA itself (van den Dobbelsteen et al., manuscript in preparation). The antibodies also showed PorA-specific bactericidal activity against the heterologous wild-type strain 2996 (B:2b:P1.5,2), however, the titres were generally lower than against isogenic TR52.

The hexavalent PorA OMV vaccine was capable of boosting the SBA responses in the infant monkeys. This was also seen in infants in the UK, where three

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doses of the hexavalent PorA OMV vaccine induced good bactericidal immune responses to two of six meningococcal strains of the vaccine. Following a fourth dose, larger bactericidal responses to all six strains were observed, suggesting that the initial course had primed memory lymphocytes or a booster response in these infants [22].

The bactericidal activity induced by the Norwegian OMV vaccine was particularly directed against PorA P1.7,16, but some additional bactericidal activity was observed. Two monkeys showed some bactericidal activity to H15 and B:15:P1.5,2. This activity could be directed against the serotype 15 protein or other surface components. The Norwegian vaccine did not induce any bactericidal reactivity against wild-type strain 2996 (B:2b:P1.5,2), although antibodies were detected in Elisa. The results obtained with the Norwegian vaccine differ somewhat from the clinical trial published by Rosenqvist et al. [7]. Heterologous bactericidal activity induced by the Norwegian OMV vaccine after three doses in adults was observed, but the surface components responsible for this activity have not been identified but may include LPS, Opa and Opc proteins or other bacterial epitopes.

The bactericidal titres against the isogenic strain B:15:P1.7,16 induced by the Norwegian OMV vaccine were generally higher than for the Dutch OMV vaccine. As the Dutch OMV vaccine contains twice the amount of PorA compared to the Norwegian vaccine (15 µg vs. 7-8 µg), this indicates that single PorA OMVs might be more immunogenic compared to triple PorA vaccines [13,17]. Alternatively, the presentation of PorA in the conventional OMV formulation might be more optimal due to the wild-type LPS, presence of PorB or other membrane components. Compared to the P1.7,16 responses for the Norwegian vaccine, equally high titres were observed for the immunodominant PorAs P1.5,2 and P1.5,10 in the triple PorA OMVs of the Dutch vaccine. A comparative study needs to be done with "monovalent" recombinant PorA OMVs and its conventional wild-type analogue, i.e. harbouring the same PorA sero-subtype, to elucidate this question.

Meningococcal PorA OMV vaccines appear to induce strong immune responses after the first dose; relatively weak booster responses were found in this and another study [21]. These characteristics probably relate to the intrinsic B cell mitogenic properties of the outer membrane proteins. This phenomenon was also noted when meningococcal OMP were used as carrier for *H. influenzae* type b polysaccharide [25].

This study clearly shows the potential of a genetically constructed hexavalent PorA OMV vaccine, which induces a bactericidal immune response against the majority of meningococcal sero-subtypes. The antibodies were still detectable after 40 weeks and the

immune response could be boosted at that time. Furthermore, a recombinant approach allows the benefit of the absence of host-identical components such as B polysaccharide and LPS and the absence of irrelevant components not contributing to the induction of functional antibodies.

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Differences in Surface Expression of NspA among *Neisseria meningitidis* Group B Strains

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NspA is a highly conserved membrane protein that is reported to elicit protective antibody responses against *Neisseria meningitidis* serogroups A, B and C in mice (D. Martin, N. Cadieux, J. Hanel, and B. R. Brodeur, J. Exp. Med. 185:1173-1183, 1997). To investigate the vaccine potential of NspA, we produced mouse anti-recombinant NspA (rNspA) antisera, which were used to evaluate the accessibility of NspA epitopes on the surface of different serogroup B strains by an immunofluorescence flow cytometric assay and by susceptibility to antibody-dependent, complement-mediated bacteriolysis. Among 17 genetically diverse strains tested, 11 (65%) were positive for NspA cell surface epitopes and 6 (35%) were negative. All six negative strains also were resistant to bactericidal activity induced by the anti-rNspA antiserum. In contrast, of the 11 NspA surface-positive strains, 8 (73%; $P < 0.05$) were killed by the antiserum and complement. In infant rats challenged with one of these eight strains, the anti-rNspA antiserum conferred protection against bacteremia, whereas the antiserum failed to protect rats challenged by one of the six NspA cell surface-negative strains. Neither NspA expression nor protein sequence accounted for differences in NspA surface accessibility, since all six negative strains expressed NspA in outer membrane preparations and since their predicted NspA amino acid sequences were 99 to 100% identical to those of three representative positive strains. However, the six NspA cell surface-negative strains produced, on average, larger amounts of group B polysaccharide than did the 11 positive strains (reciprocal geometric mean titers, 676 and 224, respectively; $P < 0.05$), which suggests that the capsule may limit the accessibility of NspA surface epitopes. Given these strain differences in NspA surface accessibility, an rNspA-based meningococcal B vaccine may have to be supplemented by additional antigens.

With the control of *Haemophilus influenzae* type b disease by vaccination, *Neisseria meningitidis* has emerged as the most common cause of bacterial meningitis in children and young adults (45, 56). Strains of *N. meningitidis* can be divided into 12 serogroups based on chemically and antigenically distinctive polysaccharide capsules (14). Five serogroups, designated A, B, C, Y, and W-135, account for virtually all disease-producing isolates (67).

Plain meningococcal polysaccharide vaccines are currently available for the prevention of disease caused by serogroups A, C, Y, and W-135 (18). These vaccines are efficacious in older children and adults but not in infants, the age group at greatest risk of acquiring the disease (18). Second-generation polysaccharide-protein conjugate vaccines are in various stages of clinical development. These vaccines are much more immunogenic in infants than are plain polysaccharide vaccines (16, 28, 32). It is likely, therefore, that highly effective meningococcal conjugate vaccines for the prevention of disease caused by serogroups A, C, Y, and W-135 strains will be licensed for routine use in children in the near future. However, attempts to develop a vaccine for the prevention of group B meningococcal disease have been problematic. Group B strains are a common cause of disease, currently accounting for approximately one-third of disease-producing isolates in the United States (10, 11), half in the United Kingdom (49), and up to 80 to 90% in The Netherlands (55). Therefore, lack of an effective vaccine for the prevention of serogroup B disease will substantially limit the overall effectiveness of a vaccination program for control of meningococcal disease.

To date, experimental meningococcal B vaccines have been designed to elicit serum antibody responses either to the group B capsule or to noncapsular antigens. Capsule-based vaccines are limited by poor immunogenicity, even when the polysaccharide is conjugated to a protein carrier (15, 26). The group B polysaccharide consists of $\alpha(2 \rightarrow 8)$ -N-acetylneuraminic acid (polysialic acid). The poor immunogenicity of this polysaccharide is attributed to immunologic tolerance as a result of exposure to cross-reacting polysialic acid expressed by a variety of host tissues, particularly the neural cell adhesion molecule (17, 23). In an innovative strategy for overcoming immunologic tolerance to the meningococcal B capsule, Jennings et al. substituted N-propionyl (N-Pr) groups for N-acetyl groups and conjugated the N-Pr meningococcal B polysaccharide derivative to a carrier protein (25). The resulting conjugate vaccine was highly immunogenic in experimental animals, eliciting immunoglobulin G (IgG) antibodies that activated complement-mediated bacteriolysis in vitro and passively protected experimental animals infected with meningococcal B bacteria. However, a subset of the antibodies elicited by this vaccine were also autoantibodies (20, 21). Given the unknown risks of a vaccine that elicits anticapsular antibodies that are also autoantibodies, recent efforts to develop a vaccine for prevention of serogroup B disease have focused on the use of alternative noncapsular antigens as vaccine components.

Non-capsular-antigen-based vaccines include outer membrane vesicles (5, 7, 42, 59, 68), specific outer membrane proteins such as PorA (6, 12, 24, 40, 41, 51, 62, 63), iron-regulated proteins such as transferrin binding protein (3, 31, 43), outer membrane opacity proteins such as Opa and Opc (39, 50), and detoxified lipopolysaccharide (64).

While vaccines based on outer membrane vesicles or recombinant PorA appear to be safe and can induce protective antibody in humans (18, 59), the bactericidal antibody responses

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TABLE 1. Reactivity of anti-rNspA polyclonal antiserum with NspA exposed on the surface of live, encapsulated, *N. meningitidis* serogroup B bacteria in relation to susceptibility to bacteriolysis and capsule production

<i>N. meningitidis</i> serogroup B ^a					NspA cell surface reactivity ^d	Anti-rNspA bactericidal activity ^e (reciprocal titer)	Polysaccharide capsule production (reciprocal titer) ^f
Strain	Country	Yr	Serologic classification ^c	ET complex			
SWZ107 ^b	Switzerland	1980	4:P1.2	104	Positive	≥64	28 ± 4
NG6/88 ^b	Norway	1988	NT: P1.1	173	Positive	4	115 ± 23
CU385	Cuba	1980	4: P1.15	5	Positive	4	116 ± 1
IH5341	Finland	1985	15:P1.7,16	ND	Positive	16	176 ± 61
BZ198	The Netherlands	1986	NT: P~	154	Positive	≥64	362 ± 1
NMB	United States	1968	Zb:P1.2,5	ND	Positive	16	244 ± 20
8047	United States	1978	Zb: P1.2	ND	Positive	16	1,125 ± 50
H44/76	Norway	1976	15: P1.7, 16	5	Positive	24	99 ± 16
1000 ^b	USSR	1989	NT:P1.5	61	Positive	<4	287 ± 12
S3446	United States	1972	14: P1.23, 14	11 (A1 cluster)	Positive	<4 (static=16)	585 ± 151
H355	Norway	1973	15: P1.15	11 cluster	Positive	<4 (static=4)	656 ± 141
BZ232 ^b	The Netherlands	1964	NT:P1.2	76	Negative	<4	1,493 ± 18
NG3/88 ^b	Norway	1988	8: P1.1	A4 cluster	Negative	<4	498 ± 105
MC58	United Kingdom	1985	15:P1.7,16b	5	Negative	<4	627 ± 121
M136	United States	1968	11: P1.15	D1 cluster	Negative	<4	1,056 ± 81
M986	United States	1963	2a: P1.5, 2	B2 cluster	Negative	<4	1,442 ± 206
NGP165	Norway	1974	NT:P1.2	37	Negative	<4	138 ± 6

^a Strains were obtained from the collection of C. Franch, Center for Biological Evaluation and Research, Rockville, Md.; 8047 was obtained from W. Zollinger, Walter Reed Army Institute of Research, Washington, D.C.; MC58 is the strain selected by The Institute of Genomic Research for genomic sequencing; IH5341 was obtained from M. Sarvas, National Public Health Institute, Helsinki, Finland; and the remaining strains are from the collection described by Seiler et al. (57). ET data are from Caugant et al. (9) and Seiler et al. (57).

^b Strain has been characterized further by multilocus sequence typing (33).

^c See reference 19 for a definition of the meningococcal serotype and subtype classification system.

^d By indirect-fluorescence flow cytometry with anti-rNspA antisera.

^e Dilution of anti-rNspA antisera that when incubated for 60 min with bacterial cells and 20% human complement yielded a 50% decrease in CFU per milliliter compared to that at time zero. "Static" refers to strains that were inhibited but not killed in the assay (≥50% but <100% survival at 60 min.).

^f Titer defined as dilution of capsule extract giving 50% inhibition of antibody binding to meningococcal B polysaccharide antigen in an ELISA (see Materials and Methods). Results are means and standard errors.

are strain specific, especially in infants (18, 59). Therefore, a multivalent PorA approach is required. In initial clinical studies of one multivalent vaccine, an outer membrane vesicle vaccine containing multiple PorA recombinant proteins, only modest serum bactericidal titers were elicited in infants, and there appeared to be antigenic interference between some of the serotype antigens (52). Given the propensity for meningococcal disease during nonepidemic periods to be caused by multiple strains or strain variants (53), together with the frequent temporal shifts in the predominant strains in a community, it will be difficult to develop a universal meningococcal B vaccine with only outer membrane protein components that are highly variable from strain to strain.

Recently, Martin et al. described a membrane protein, designated neisserial surface protein A (NspA), which was reported to be highly conserved across *Neisseria* strains (36) and elicited serum bactericidal antibody responses in mice to *N. meningitidis* strains from serogroups A, B, and C (36). Immunized mice also were reported to be protected from a lethal challenge with live meningococcal B organisms (36). Thus, NspA appears to represent a novel and promising vaccine candidate.

To investigate further the vaccine potential of NspA, we cloned the *nspA* gene, expressed NspA in *Escherichia coli*, and produced mouse polyclonal anti-recombinant NspA (anti-rNspA) antisera. The resulting antisera were used to evaluate the accessibility of NspA epitopes on the surface of different strains of live encapsulated *N. meningitidis* group B bacteria and the susceptibility of these strains to antibody-dependent, complement-mediated bacteriolysis. In this report, we confirm that the NspA gene and protein sequences are highly conserved among genetically divergent strains of *N. meningitidis* group B. However, despite conservation of the protein se-

quence and expression, we found strain differences in the surface accessibility of NspA epitopes and in the susceptibility of different strains to anti-NspA bactericidal activity.

MATERIALS AND METHODS

Bacterial strains. The *N. meningitidis* strains studied were isolated from patients residing in different countries over a period of more than 30 years (Table 1). The specific strains were selected to be representative of widely divergent "clone" groups, as defined by multilocus isoenzyme typing and/or multilocus sequence typing (33, 57). Strain M7, which is derived from strain NMB, contains a transposon insertion that blocks capsular polysaccharide biosynthesis (58). All of the other strains were encapsulated.

Cloning, expression, and purification of rNspA and HisTag-NspA. Based on the nucleotide sequence published by Martin et al. (36), the following primers that are complementary to opposing strands at the 5' and 3' ends of the *nspA* gene, respectively, were synthesized: 5'-ACAGCAGGATCCTTAACGGATT C-3' and 5'-GTGGATGAAGCCTTTGGACATTTC-3'. In addition, the primers contained base substitutions that created cleavage sites for *Bam*HI and *Hind*III at the 5' and 3' ends, respectively, of the *nspA* gene. By using the method described for DNA sequencing below, these primers were used in PCR to amplify a 743-bp DNA segment from the genome of *N. meningitidis* 8047. The fragment, which includes the wild-type promoter region, was subsequently cloned into the multicopy plasmid pSK(+) (Stratagene, San Diego, Calif.). In addition, we obtained as a gift from R. Rappunil (Chiron Vaccines, Siena, Italy) a plasmid that contains a truncated version of the *nspA* gene (pTrc.NspA.1). In this construct, the portion of the gene encoding the signal sequence has been replaced with DNA encoding a series of six histidine residues (expression vector pTrcHis [Invitrogen, Carlsbad, Calif.]).

Recombinant HisTag-NspA was expressed in *Escherichia coli* BL21(DE3) (Stratagene) transformed with plasmid pTrc.NspA.1 by induction with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). HisTag-NspA protein was purified by Ni-nitrilotriacetic acid Sepharose chromatography of cell lysates solubilized in 6 M guanidine hydrochloride by using the materials and methods provided in the QiaExpress kit (Qiagen, Valencia, Calif.). Recombinant wild-type NspA, expressed under the control of its own promoter from plasmid pGMS 1.0, was also produced in *E. coli* BL21(DE3). The protein was excreted into the culture medium and was partially purified by precipitation of culture medium with ammonium sulfate (55%, wt/vol).

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DNA preparation and sequencing. Multiple and single clones of each strain were grown to an absorbance at 620 nm (A_{620}) of 1 in 7 ml of Mueller-Hinton broth (Difco, Detroit, Mich.) supplemented with 0.2% glucose. Genomic DNA was prepared with a commercial kit (Qiagen) as specified by the manufacturer for the preparation of genomic DNA from bacteria (47). DNA used for sequencing was obtained by PCR amplification of *nspA* in the genomic DNA preparation with the primers described above. The *Taq* polymerase and other reagents used for PCR were from Qiagen. The protocol consisted of a denaturation step (94°C for 5 min) followed by 35 cycles of amplification (94°C for 1 min, 60°C for 2 min, and 72°C for 1.5 min) and 1 cycle of extension (72°C for 5 min). Amplified DNA was purified with a QIAquick PCR purification kit (Qiagen) as specified by the manufacturer (48). DNA sequencing of each clone was performed by McConnel Research (San Diego, Calif.) with the primers given above. For determination of the sequence of the *nspA* gene of each strain, two or three independent PCR products from single clones were sequenced on both strands. Sequence alignments and translations were performed with MacVector software (Oxford Molecular, Cambridge, Mass.).

Polyclonal anti-rNspA antisera. To prepare antisera against NspA, 50 μ g of purified HisTag-NspA or partially purified wild-type rNspA expressed from its own promoter was used to immunize groups of 4- to 6-week-old female CD-1 mice (five to eight mice per group). Injections were given intraperitoneally (i.p.). Complete Freund's adjuvant was used for the first dose, and incomplete Freund's adjuvant was used for two subsequent booster doses given at 2- to 3-week intervals. As controls, groups of mice were immunized with the same adjuvants combined with proteins precipitated from culture medium supernatant of *E. coli* BL21(DE3) cells transformed with the parent plasmid pSK(+), which lacks the *nspA* gene. In a few experiments, we also immunized mice intramuscularly with 50 μ g of rNspA given with 5 μ g of purified QuilA (gift of G. Ott, Chiron Corp., Emeryville, Calif.) as the adjuvant, and we also compared the responses of female BALB/c and CD-1 mice. For assessment of serum antibody responses, individual mouse sera obtained 2 to 4 weeks after the third immunization were pooled.

Binding of antisera to the surface of live encapsulated meningococci. The ability of antisera elicited by the recombinant NspA constructs to bind to the surface of pathogenic strains of *N. meningitidis* group B was determined by flow cytometric detection of indirect fluorescence assay, performed as described previously (21). In brief, bacterial cells were grown to mid-log phase in Mueller-Hinton broth, harvested by centrifugation, and resuspended in blocking buffer (phosphate-buffered saline [PBS] containing 1% [wt/vol] bovine serum albumin and 0.2% [wt/vol] sodium azide) at a density of $\sim 10^8$ cells per ml. Dilutions of test or control antiserum (typically 1:10 to 1:100) were then added and allowed to bind to the cells, which were maintained on ice for 2 h. Following two washes with blocking buffer, the cells were incubated with the fluorescein isothiocyanate-conjugated F(ab')₂ fragment of goat anti-mouse IgG (heavy plus light chains) (Jackson ImmunoResearch, West Grove, Pa.) and fixed with 0.25% formaldehyde in PBS buffer, and the bacterial cells were analyzed by flow cytometry.

Positive control antibodies included meningococcus-specific serotyping or subtyping monoclonal antibodies (MN2C3B and MN16C13F4 [Rijksinstituut voor Volksgezondheid en Milieu, Bilthoven, The Netherlands] and SEAM 12, an anti-polysaccharide monoclonal antibody (MAb) that is specific for encapsulated group B strains (21). The negative control consisted of a mouse IgG MAb (VIG10) of irrelevant specificity.

Complement-dependent bactericidal antibody activity. The bactericidal assay was adapted from the method previously described from this laboratory (35) with the following modifications. After overnight growth on chocolate agar, several colonies were inoculated into Mueller-Hinton broth (starting A_{620} of ~ 0.1) and the test organism was grown for approximately 2 h to an A_{620} of ~ 0.6 . After the bacteria were washed twice in Gey's buffer, approximately 300 to 400 CFU was added to the reaction mixture. The final reaction mixture of 60 μ l contained 20% (vol/vol) complement and serial twofold dilutions of test sera or control MAbs in Gey's buffer (instead of barbital buffer as previously described by Mandrell et al. [35]). The complement source was human serum from a healthy adult with no detectable anticapsular antibody to group B polysaccharide when tested by the enzyme-linked immunosorbent assay (ELISA) (22) and no detectable intrinsic bactericidal activity at a final concentration of 20 or 40% against the test strain. In preliminary experiments with a panel of test sera, this complement source gave comparable bactericidal titers to those obtained with agammaglobulinemic serum as the complement source. Serum bactericidal titers were defined as the serum dilution (or antibody concentration) resulting in a 50% decrease in CFU per milliliter after a 60-min incubation of bacteria in the reaction mixture, compared to the control CFU per milliliter at time zero. Typically, bacteria incubated with the negative control antibody and complement showed a 150 to 200% increase in CFU per milliliter during the 60 min of incubation.

Animal protection. The ability of the anti-rNspA antiserum to confer passive protection against *N. meningitidis* group B bacteremia was tested in infant rats challenged i.p. by a method adapted from that of Sankkonen et al. (54). In brief, 6- to 7-day-old pups from six litters of outbred Wistar rats (Charles River, Hollister, Calif.) were randomly redistributed to the nursing mothers. Groups of five or six animals were challenged i.p. with 100 μ l of approximately 5×10^6 CFU of *N. meningitidis* group B bacteria. Two strains, M986 and 8047, each of which had been passaged three times in infant rats, were tested. The bacteria isolated from blood cultures after the third pass were grown on chocolate agar overnight

and stored frozen at -70°C in vials containing sterile skim milk. On the day of the experiment, the bacteria were grown, washed, and resuspended in Gey's buffer, as described above for the bactericidal assay. Immediately before administration to the animals, the bacterial suspension was mixed with different dilutions of test or control antiserum or MAb. At 18 h after the bacterial challenge, blood specimens were obtained by puncturing the heart with a syringe and needle containing approximately 25 U of heparin without preservative (Fujisawa USA, Deerfield, Ill.). Aliquots of 1, 10, and 100 μ l of blood were plated onto chocolate agar. The CFU per milliliter of blood was determined after overnight incubation of the plates at 37°C in 5% CO_2 .

Membrane preparations. Single colonies were grown to an A_{620} of 0.7 to 0.9 in 7 ml of Mueller-Hinton broth (Difco) supplemented with 0.2% glucose. The 7-ml culture was then used to inoculate 200 ml of the same medium prewarmed to 37°C . After growth of the culture to an A_{620} of 0.9, the bacteria were collected by centrifugation at $5,000 \times g$ for 15 min. The cell pellets were frozen at -20°C until used for preparation of membrane proteins. Sodium lauryl sarcosinate-insoluble extracts of bacterial membranes were prepared as described previously for *H. influenzae* type b (4). Briefly, the cell pellets were resuspended in 10 ml of HEPES buffer (pH 7.4) at 4°C . The cell suspension was then sonicated on ice with several 15-s bursts from a microprobe sonifier (Branson, Danbury, Conn.). Cell debris was removed by centrifugation at $5,000 \times g$ for 20 min, and the membrane vesicles in the supernatant were obtained by ultracentrifugation at $100,000 \times g$ for 1 h at 4°C . The resulting membrane pellet was resuspended in 2 ml of HEPES buffer (pH 7.4) containing 1% (wt/vol) sodium lauryl sarcosinate (Sigma, St. Louis, Mo.) at ambient temperature. After a 30-min incubation, the sodium lauryl sarcosinate-insoluble fraction was collected by ultracentrifugation at $100,000 \times g$ for 2 h.

SDS-PAGE and Western blots. NspA protein preparations were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (21% polyacrylamide) as described by Laemmli (27) with a Mini-Protein II electrophoresis apparatus (Bio-Rad, Richmond, Calif.). Samples were suspended in SDS sample buffer (0.05 M Tris-HCl [pH 6.8], 10% [vol/vol] glycerol, 2% [wt/vol] SDS, 5% [vol/vol] 2-mercaptoethanol, 10 μ g of bromophenol blue per ml) and either heated to 100°C for 5 min or, in some experiments, left at ambient temperature for 5 min before being loaded directly onto the gel. For Western blots, the gel was equilibrated with buffer (48 mM Tris-HCl, 39 mM glycine [pH 9.0], 20% [vol/vol] methanol) and transferred to a nitrocellulose membrane (Bio-Rad) by using a Trans-Blot (Bio-Rad) semidry electrophoretic transfer cell. The nitrocellulose membranes were blocked with 2% (wt/vol) skim milk in PBS containing 0.2% (wt/vol) sodium azide. Anti-HisTag-NspA antisera were diluted in PBS containing 1% (wt/vol) bovine serum albumin, 1% (wt/vol) Tween 20, and 0.2% (wt/vol) sodium azide. Bound antibody was detected with rabbit anti-mouse IgG-, IgA-, and IgM-alkaline phosphatase conjugate polyclonal antibody (Zymed, South San Francisco, Calif.) and Sigma Fast BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium) substrate (Sigma).

Quantitation of capsular polysaccharide antigens. The amount of capsular polysaccharide produced by each strain was determined by an inhibition ELISA. First, extracts of capsular polysaccharide were prepared based on a method described by Corn et al. (13). In this method, individual bacterial clones were grown for 1.5 to 2 h in 7 ml of Mueller-Hinton broth to an A_{620} of 0.5 to 0.7. The bacteria were collected by centrifugation at $5,000 \times g$ for 15 min. The cells were washed once in 0.6 ml of 10 mM HEPES (pH 8.0) and then resuspended in 0.6 ml of the same buffer containing 10 mM EDTA and incubated at 37°C for 1 h. The cells were pelleted at $10,000 \times g$ for 1 min, and the relative amount of meningococcal B polysaccharide antigen released into the supernatant was determined by an inhibition ELISA, performed as described by Anni et al. (2). The solid-phase antigen in the ELISA was meningococcal B polysaccharide-*adipic dihydrazide*-biotin absorbed to avidin-coated microtiter plates as previously described (22). The meningococcal B polysaccharide-reactive human paraprotein LIP (7), at concentration of 0.2 $\mu\text{g/ml}$, was used as the primary antibody. In the absence of inhibitor, this concentration of antibody was sufficient to give an A_{492} of ~ 0.7 to 1.0 after a 30-min incubation with substrate (2). The titer of polysaccharide released into the supernatant was measured by determining the dilution of supernatant that resulted in 50% inhibition of antibody binding. Controls in this assay included an EDTA extract prepared from strain M7, which does not produce any capsular polysaccharide (58), and purified meningococcal B polysaccharide. To ensure that all of the capsular polysaccharide was released by the EDTA treatment, the same inhibition ELISA was performed with the cell pellet resuspended in the same buffer and volume as the capsule extract. The observable inhibitory activity from the cell pellet was between 0 and 10% of the activity observed in the capsule extracts, with the latter, higher percentages coming from cell pellets of strains that produce the largest amounts of capsule.

RESULTS

Figure 1A shows a Coomassie blue-stained SDS-PAGE gel (21% polyacrylamide). The sample in lane 1 contains the HisTag-NspA, purified by Ni-nitrilotriacetic acid Sepharose chromatography of cell lysates solubilized in 6 M guanidine

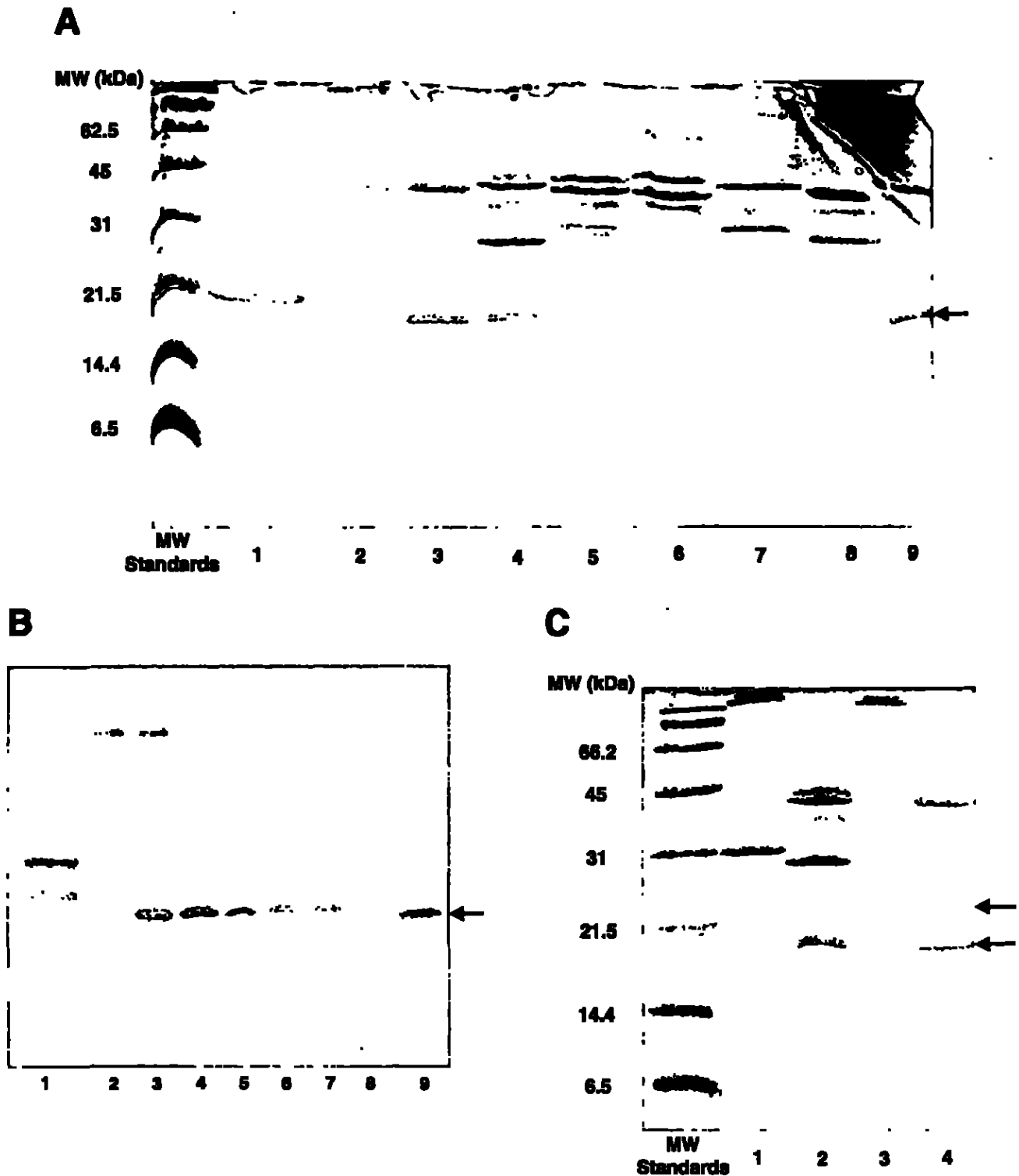


FIG. 1. (A) Coomassie blue-stained SDS-PAGE gel (21% polyacrylamide). Lanes: 1, HisTag-NspA eluted from the Ni-NTA Sepharose metal affinity column with 8 M urea-0.1 M NaH_2PO_4 -0.01 M Tris-HCl buffer at pH 4.5; 2, ammonium sulfate-precipitated culture medium from *E. coli* BL21(DE3) transformed with pSK(+) (the parent plasmid without the *nspA* gene); 3 and 9, ammonium sulfate-precipitated culture medium from *E. coli* BL21(DE3) transformed with pGMS1.0 [the pSK(+) plasmid containing the wild-type *nspA* gene without the His tag]; production of rNspA is evident by the appearance of a protein with an apparent molecular mass of 18.6 kDa (arrow); 4 to 8, lauryl sarcosinate-insoluble outer membrane preparations of meningococcal B strains 8047 (lane 4), CU385 (lane 5), NG6/88 (lane 6), M966 (lane 7), and M136 (lane 8). (B) Blotting of anti-HisTag-NspA antiserum as shown by Western blotting. Lanes are identical to those in panel A. Except for HisTag-NspA, which appears as two immunoreactive bands at higher apparent molecular masses (see the text), the presence of NspA in each of the samples is indicated by the arrow at an apparent molecular mass of 18.6 kDa. (C) Coomassie blue-stained SDS-PAGE gel (21% polyacrylamide). Lanes: 1, lauryl sarcosinate-insoluble membrane proteins from strain 8047 that has not been heated in sample buffer; 2, sample comparable to that in lane 1 that was heated to 100°C for 5 min; 3, ammonium sulfate-precipitated culture medium from *E. coli* BL21(DE3) transformed with pGMS 1.0 [the pSK(+) plasmid containing the wild-type *nspA* gene without the His tag] that has not been heated in sample buffer; 4, a sample comparable to that in lane 3 that was heated to 100°C for 5 min. The arrows indicate the shift from the higher apparent molecular weight to the expected molecular weight of NspA, as a result of heating the sample in sample buffer.

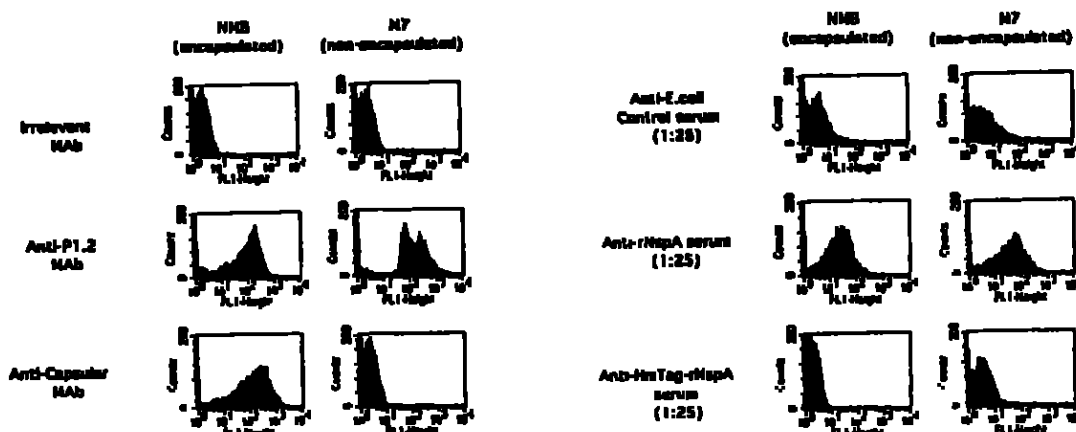


FIG. 2. Binding of polyclonal anti-rNspA antisera and control MAbs to encapsulated and nonencapsulated strains of live meningococcal B bacteria as determined by indirect-fluorescence flow cytometry. Strain M7 is a nonencapsulated mutant derived from strain NMB by transposon insertion (58). All antisera were tested at a dilution of 1:25. The control MAbs and antisera include VIG10, a murine MAb with an irrelevant specificity; *N. meningitidis* subtype MAb anti-P1.2 reagent (Rijksinstituut Voor Volksgezondheid en Milieu, Bilthoven, The Netherlands); and SEAM 3, an anti-capsule-specific murine MAb (21); as well as pooled sera from mice immunized with proteins from the supernatant culture of *E. coli* BL21(DE3), containing the vector that lacks the *nspA* gene (anti-*E. coli* control). The last antisera include anti-rNspA antisera elicited by immunization of mice with ammonium sulfate-precipitated culture medium from *E. coli* BL21(DE3) that expresses rNspA from plasmid pGMS 1.0 and polyclonal anti-HisTag-NspA antiserum.

hydrochloride, which resolves as two bands with apparent molecular masses of 20.2 and 24.5 kDa. These bands appear to be different forms of HisTag-NspA rather than different proteins, since both the number and the apparent molecular masses of the bands in the HisTag-NspA sample are dependent on the content of SDS and EDTA in the sample buffer (data not shown).

According to Martin et al. (36), NspA is associated with the outer membrane. However, recombinant NspA produced in *E. coli* (without the His tag) is secreted into the culture medium. In their experiments, the "secreted" fraction of the rNspA protein was affinity purified and used as a vaccine. Figure 1A, lane 2, shows the proteins present in ammonium sulfate-precipitated culture medium from *E. coli* BL21(DE3) transformed with pSK(+), the parent cloning vector without the *nspA* gene. Lanes 3 and 9 contain duplicate samples of the ammonium sulfate-precipitated culture medium from the same *E. coli* strain transformed with plasmid pGMS1.0, which contains the wild-type *nspA* gene. The presence of rNspA is evident by the appearance of a protein with an apparent molecular mass of 18.6 kDa, corresponding to the molecular mass of NspA predicted from the amino acid sequence. This protein is not present in the control (lane 2). Lanes 4 to 8 contain sodium lauroyl sarcosinate-insoluble membrane proteins from strains 8047, CU385, NG6/88, M986, and M136, respectively. All contain a protein having the same mobility (18.6 kDa) as rNspA (lanes 3 and 9).

Confirmation that the 18.6-kDa protein observed in Fig. 1A, lanes 3 to 9, is NspA was obtained by Western blot analysis with anti-HisTag-NspA polyclonal serum as the detecting antibody. The results are shown in Fig. 1B. The 18.6-kDa bands present in Fig. 1B, lanes 3 to 9, are reactive with the anti-HisTag-NspA sera. Note that in lanes 2 and 3 the anti-HisTag NspA antisera also react with higher-molecular-weight proteins. However, these cross-reacting higher-molecular-weight proteins were present in both the control (i.e., without NspA) and rNspA-containing preparations.

When analyzed by SDS-PAGE, NspA is reported to have two forms, a 22-kDa diffuse band and a 18-kDa band (36). However, we found that these two forms resulted because

NspA is a heat-modifiable protein. Figure 1C shows an SDS-PAGE gel (21% polyacrylamide) of sodium lauroyl sarcosinate-insoluble outer membrane proteins from strain 8047 and partially purified rNspA. Lane 3 contains rNspA that has not been heated in sample buffer. A diffuse band with an apparent molecular mass of 23 kDa is present. Although not shown, a comparable band was not present in proteins present in ammonium sulfate-precipitated culture medium from *E. coli* containing the parent cloning vector without the *nspA* gene. As shown in Fig. 1C, lane 4, when the partially purified rNspA was heated to 100°C in sample buffer, the 23-kDa band disappeared and was replaced by a protein with an apparent molecular mass of 18.6 kDa. A similar shift in mobility was observed in the respective proteins present in the detergent-insoluble outer membrane protein preparation from strain 8047 (compare lanes 1 and 2). In both the rNspA preparation and the outer membrane preparation from strain 8047, the 18.6-kDa band was recognized by the anti-HisTag-NspA antiserum in the Western blot (Fig. 1B). The anti-HisTag NspA antiserum also recognized the respective 23-kDa bands in samples that had not been heated (data not shown).

Binding of anti-rNspA serum to native NspA on the surface of live meningococcal B bacteria. Binding of serum antibodies to the cell surface of live meningococci was measured by flow cytometric detection of indirect immunofluorescence (21). Figure 2 shows the results of a typical experiment examining antibody binding to two test strains: NMB, a fully encapsulated *N. meningitidis* group B strain, and M7, a transposon-containing capsule-deficient mutant of NMB (58). As expected, the anti-group B polysaccharide MAb (SEAM 3) (21) binds only to the encapsulated strain whereas as the positive control anti-P1.2 (PorA) MAb binds to both the encapsulated strain and the nonencapsulated mutant. The ability of pooled antisera from mice immunized with rNspA to bind to bacteria is also shown. The antisera from mice immunized with wild-type rNspA show an increase in fluorescence intensity with both the encapsulated and nonencapsulated mutant strains when the antisera were tested at dilutions of 1:10 to 1:100. In contrast, polyclonal antisera prepared to proteins precipitated from culture supernatant of the *E. coli* vector alone (without the *nspA*

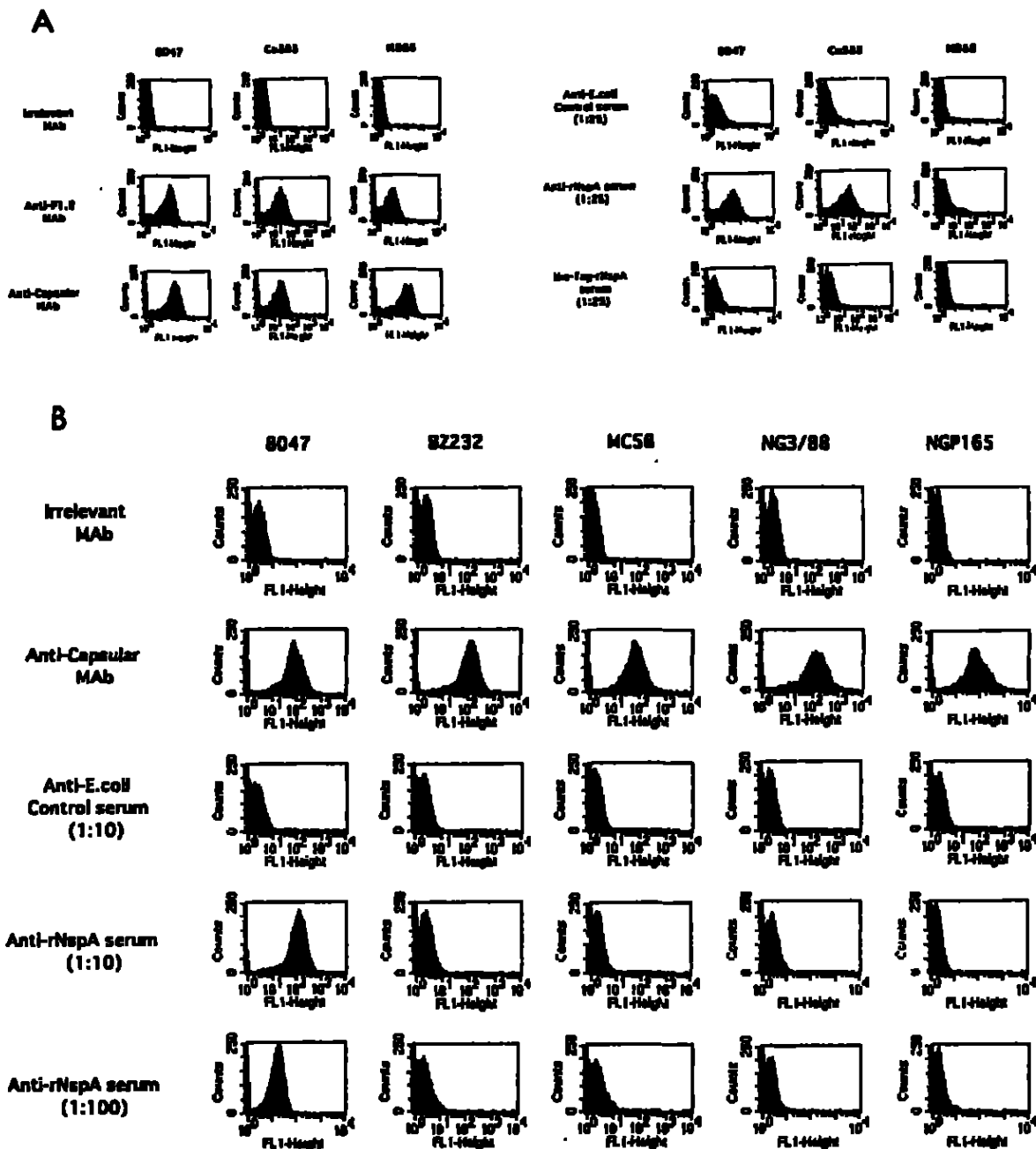


FIG. 3. (A) Binding of polyclonal anti-rNspA antiserum and control MAbs to encapsulated meningococcal B strains 8047, CU385, and M986, as determined by indirect-fluorescence flow cytometry. All sera were tested at a dilution of 1:25. The test and control MAbs and antisera are those described in the legend to Fig. 2. (B) Binding of polyclonal anti-rNspA antiserum and control MAbs to encapsulated meningococcal B strains 8047, B2232, MC58, NG3/88, and NGP165, as determined by indirect-fluorescence flow cytometry. All sera were tested at dilutions of 1:10 and 1:100 as indicated. The irrelevant and antcapsular control MAbs and control anti-E. coli antisera are those described in the legend to Fig. 2.

gene) showed only low-intensity background fluorescence and were considered negative. Note that in Fig. 2, representative data are shown only for antisera diluted 1:25.

As shown in Fig. 2, the antiserum prepared against purified, denatured HisTag-NspA gave background levels of fluorescence with strains NMB and M7 and were considered negative. This antiserum was also negative for bacterial binding with strains 8047, CU385, and M986 (Fig. 3A, right). However, by Western blotting (Fig. 1B), the anti-HisTag-NspA antiserum

recognized purified HisTag-NspA (Fig. 1B, lane 1), wild-type rNspA (lanes 3 and 9), as well as NspA present in a detergent-insoluble outer membrane fraction from four different *N. meningitidis* group B strains, including strains 8047, CU385, and M986 (lanes 4 and 8). Taken together, the data suggest that the antibodies prepared to HisTag-NspA recognize epitopes that are present in denatured NspA but not in native NspA present on the surface of live meningococcal B bacteria. In contrast, the antibodies prepared against wild-type rNspA, which were

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detected by the flow cytometric binding assay, are likely to be specific for conformational NspA epitopes.

Strain differences in NspA surface accessibility. The flow cytometric assay was used to assess the ability of pooled IgG anti-rNspA antibodies to bind to the surface of 17 genetically diverse strains of live, encapsulated pathogenic *N. meningitidis* group B bacteria isolated from patients residing in different countries over a period of more than 30 years. Figure 3A shows examples of antibody binding to the surface of *N. meningitidis* 8047, the homologous strain from which we cloned the *nspA* gene, and two heterologous strains, CU385 and M986. All three strains showed no binding with a negative control MAb of irrelevant specificity but bound strongly to two positive control MAbs, an anticapsular antibody and anti-P1.2 (left). In contrast, the anti-rNspA antibody bound only to strains CU385 and 8047 and there was no detectable anti-rNspA antibody binding to strain M986 (right).

Figure 3B shows examples of four additional strains, BZ232, MCS8, NG3/88, and NGP165, that showed no detectable anti-rNspA antibody binding to the cell surface when tested by flow cytometry at an antiserum dilution of 1:10. By comparison, strain 8047 was strongly positive when tested at dilutions of 1:10 and 1:100. All five strains also showed strong binding to the control anticapsular MAb.

As summarized in Table 1, of the 17 *N. meningitidis* group B strains tested by flow cytometry, 11 (65%) were positive for NspA cell surface binding and the remaining 6 were negative. There was no apparent relationship between cell surface NspA expression in a given strain and the respective serotype or subtype, or electrophoretic type (ET) classification, or the year or country of isolation.

Sequence variability in NspA from different strains. One possible explanation for apparent strain differences in reactivity with the anti-rNspA antisera in the flow cytometric assay is polymorphisms in the NspA protein. To test this possibility, we cloned and sequenced the *nspA* gene from five of the six negative strains (BZ232, NG3/88, NGP165, M136, and M986) and from three of the positive strains (8047, CU385, and NG6/88). The GenBank accession numbers for the *nspA* gene sequences for these strains are AF175676 to AF175683. For the sixth negative strain, MCS8, we obtained the nucleotide sequence of the *nspA* gene from data generated by The Institute of Genomic Research, as part of their MCS8 genome sequencing studies (60). The *nspA* gene sequences of all 10 strains were highly conserved. In comparison to the DNA sequence published by Martin et al. (36), there were between zero and five nucleotide differences resulting in zero to three amino acid differences. Further, as shown in Fig. 4, with one exception, all of the amino acid variants involved the same respective residues in discrete segments of the protein. These included the signal peptide, which is not present in the mature protein, and two short segments in the carboxyl-terminal 50 amino acids of the protein. Evidently, these differences are inconsequential for recognition of the protein by the anti-rNspA antisera, since there are examples of identical derived amino acid sequences of the mature NspA from strains that were negative for reactivity with anti-rNspA antisera and those that were positive (compare M136 to 8047, NGP165 to NG6/88, and MCS8 to CU385).

Detergent-insoluble outer membrane fractions prepared from different *N. meningitidis* group B strains. The indirect-fluorescence cell binding assay provides information on the surface accessibility of NspA epitopes that are likely to be important in interacting with protective antibody. Given the identical or nearly identical *nspA* genes in the different strains, the failure to detect NspA surface expression in some strains

but not others could reflect decreased amounts of NspA in the outer membrane of the former, possibly as a result of strain differences in *nspA* gene expression.

To investigate possible strain differences in NspA expression in the outer membrane, we extracted bacterial cell pellets with sodium lauroyl sarcosinate and analyzed the insoluble outer membrane fraction by SDS-PAGE and Western blotting. Data from five representative strains, three that were positive (8047, CU385, and NG6/88) and two that were negative (M986 and M136) for anti-rNspA antisera binding by the flow cytometric assay, are shown in Fig. 1. As described above, a band with an apparent molecular mass of 18.6 kDa was observed in membrane fractions from all five strains analyzed by SDS-PAGE (Fig. 1A), and this band was cross-reactive with anti-HisTag-NspA serum by Western blotting (Fig. 1B). In other experiments (results not shown), NspA was detected in sodium lauroyl sarcosinate-insoluble outer membrane fractions prepared from the remaining four strains that were negative for cell surface binding by the flow cytometric assay (strains NG3/88, MCS8, NGP165, and BZ232). NspA was also detected by Western blotting with antisera prepared to partially purified rNspA without the His tag (data not shown).

Among the strains tested, the density of the 18.6-kDa NspA band present in the OMP preparation from strain 8047 (Fig. 1A) and BZ198 (data not shown) appeared to be greater than that of the corresponding bands from the other strains. These observations were consistent in multiple membrane protein preparations from the strains.

Production of capsular polysaccharide. The ability of anti-rNspA antibody to bind to the bacterial cell surface could also be influenced by the amount of polysaccharide capsule present. To test this hypothesis, we compared the quantity of capsular polysaccharide produced by each of the strains that were scored as positive or negative for anti-NspA binding by the flow cytometric assay. As described in Materials and Methods, the bacterial cells were suspended in buffer and incubated with EDTA to release capsular polysaccharide. The amount of soluble capsule released was measured by an inhibition ELISA. The data were expressed as the reciprocal dilution of supernatant giving 50% inhibition of binding of an anticapsular MAb to biotinylated meningococcal B polysaccharide adhering to avidin-coated wells. Each strain was tested for capsular polysaccharide production in two to four independent experiments. From these results, a mean value ($\pm$ standard error) was assigned to each strain. The results for the individual strains are summarized in Table 1. Although there are a few notable outliers (e.g., strains 8047 and NGP165), on average the 6 strains that were negative for reactivity with anti-rNspA antisera by the flow cytometric assay produced threefold more capsular polysaccharide than did the 11 strains that were positive (reciprocal geometric mean dilutions of 676 and 224, respectively [$P < 0.05$ by the *t* test]). We also measured the amount of capsular polysaccharide released into the culture medium for representative NspA-positive (CU385 and H44/76) and -negative (M136 and M986) strains by the same inhibition ELISA. In each case, the relative amount of polysaccharide released by each strain into the medium correlated directly with the amount of capsule polysaccharide measured in the bacterial extracts (data not shown). Thus, the smaller amounts of cell-associated capsule in the NspA cell surface positive strains were not a result of greater shedding of capsular material into the broth.

Complement-mediated bactericidal activity of anti-rNspA antiserum. Table 1 summarizes the results of measurement of complement-mediated bactericidal activity of the anti-rNspA antiserum for each of the strains tested. All 17 strains were

	Signal Peptide				Loop 1			
	-10	1	10	20	30	40		
NM 608 B	MKKALATLIA	LALPAAALAE	GASGFYVQAD	AAHAKASSSL	GSAKGFSPRI	SAGYRINDL		
NM NG6/88 ⁺	-----	-----	-----	-----	-----	-----		
NM CU385 ⁺	-----	-----	-----	-----	-----	-----		
NM 8047 ⁺	-----A-----	-----	-----	-----	-----	-----		
NM NGP165 ⁻	-----	-----	-----	-----	-----	-----		
NM MC58 ⁻	-----	-----	-----	-----	-----	-----		
NM M986 ⁻	-----	-----	-----	-----	-----	-----		
NM BZ232 ⁻	-----	--I-----	-----	-----	-----	-----		
NM M136 ⁻	-----	--I-----	-----	-----	-----	-----		
NM NG3/88 ⁻	-----	--I-----	-----	-----	-----	-----		
NG FA1090	-----A-----	-----	-----	-----*	-----	-----		
NG B2	-----A-----	-----	-----	-----	-----	-----		
	Loop 2				Loop 3			
	50	60	70	80	90	100		
NM 608B	RFAVDYTRYK	NYKAPSTDFK	LYSIGASAIY	DFDTQSPVKP	YLGARLSLNR	ASVDLGGSDS		
NM NG6/88 ⁺	-----	-----	-----	-----	-----	-----		
NM CU385 ⁺	-----	-----	-----	-----	-----	-----		
NM 8047 ⁺	-----	-----	-----	-----	-----	-----		
NM NGP165 ⁻	-----	-----	-----	-----	-----	-----		
NM MC58 ⁻	-----	-----	-----	-----	-----	-----		
NM M986 ⁻	-----	-----	-----	-----	-----	-----		
NM BZ232 ⁻	-----	-----	-----	-----	-----	-----		
NM M136 ⁻	-----	-----	-----	-----	-----	-----		
NM NG3/88 ⁻	-----	-----	-----	-----	-----	-----		
NG FA1090	-----	--Q-----	-----V-	-----	--F-----	--AH-----		
NG B2	-----	-----	-----V-	-----	--F-----	--AH-----		
	Loop 4							
	110	120	130	140	150			
NM 608B	FSQTSIGLGV	LTGVSYAVTP	NVDLDAGYRY	NYIGKVNTVK	NVRSGELSVG	VRVKF		
NM NG6/88 ⁺	-----	-----	-----	-----	-----	-----		
NM 8047 ⁺	-----T-----	--A-----	-----	-----	-----	-----		
NM CU385 ⁺	-----	-----	-----	-----	-----A	-----		
NM NGP165 ⁻	-----	-----	-----	-----	-----	-----		
NM MC58 ⁻	-----	-----	-----	-----	-----A	-----		
NM M986 ⁻	-----	-----	-----	-----	-----	-----		
NM BZ232 ⁻	-----T-----	-----	-----	-----	-----A	-----		
NM M136 ⁻	-----T-----	--A-----	-----	-----	-----	-----		
NM NG3/88 ⁻	-----T-----	--A-----	-----	-----	-----A	-----		
NG FA1090	---K--A---	--A-----	-----	---V-----	-----A	-----		
NG B2	---K--A---	--A-----	-----	---V-----	-----A	-----		

FIG. 4. Predicted amino acid sequences of NspA from different *N. meningitidis* serogroup B strains. Sequencing data for *N. meningitidis* 608B were obtained from Martin et al. (36) (GenBank accession no. U52066), and those for strain MC58 were obtained from The Institute for Genome Research (49a). Data for *N. gonorrhoeae* B2 were from GenBank (accession no. U52069), and data for FA1090 were from the University of Oklahoma *Neisseria gonorrhoeae* Genome Sequencing Project. The numbering corresponds to that of the mature protein. Underlined segments are putative surface-exposed loops (see Fig. 5). NM indicates that the NspA sequence is from *N. meningitidis*, and NG indicates that it is from *N. gonorrhoeae*. The superscript + and - indicate strains that were positive or negative, respectively, for anti-rNspA antiserum binding as determined by the flow cytometric assay.

killed by complement together with similar concentrations of a positive control anti-capsular MAb (SEAM 12; subtype IgG2a [21]). In contrast, the six strains that were negative for anti-rNspA antiserum binding by the flow cytometric assay were resistant to anti-rNspA antibody-induced complement-mediated bactericidal or bacteriostatic activity (each showed a 150 to 200% increase in CFU per milliliter compared to the control CFU per milliliter at time zero when assayed at the lowest dilution of the anti-NspA antiserum tested, 1:4).

Of the 11 strains that were positive for NspA surface expression by flow cytometry, 8 (73%) (SWZ107, IH5341, CU385, NG6/88, BZ198, H44/76, NMB, and 8047) were killed by complement and the anti-rNspA antiserum and 3 were not killed (strains 1000, H355, and S3446). Of interest, with the exception of strain 1000, growth of the other two NspA cell surface-positive strains that were not killed in the assay was inhibited by the anti-rNspA antiserum and complement (survival after a 60-min incubation was less than 100% but greater than 50%,

TABLE 2. Protective activity of anti-rNspA antisera^a

Treatment	Dose/rat or serum dilution	Strain	Blood culture		
			No. positive/total no.	10 ³ mean CFU/ml	CFU/ml (% of control) ^d
Anticapsular MAb	2 µg	M986	0/6	<1	<1
Anti-rNspA	1:5	M986	6/6	44 ^c	45
Anti-rNspA	1:25	M986	6/6	93 ^c	95
Anti- <i>E. coli</i> negative control	1:5	M986	6/6	98 ^c	
Anticapsular MAb	2 µg	8047	0/5	<1	<1
Anti-rNspA	1:5	8047	1/6	0.2 ^d	2
Anti-rNspA	1:25	8047	1/5	0.4 ^d	4
Anti- <i>E. coli</i> negative control	1:5	8047	6/6	10 ^d	

^a Infant rats (6 days old) were challenged i.p. with 100 µl of buffer containing 5.4×10^5 CFU of strain M986 (negative for NspA surface-accessible epitopes) or 6.3×10^5 CFU of strain 8047 (positive for NspA surface-accessible epitopes) together with antiserum or MAb. Quantitative blood cultures were obtained 18 h later. For calculation of geometric mean CFU per milliliter, animals with sterile cultures were assigned values of 0.1 CFU/ml.

^b Calculated in comparison to geometric mean CFU per milliliter for animals challenged with the respective strain.

^c $P > 0.5$ compared to geometric mean CFU per milliliter for respective control rats.

^d $P < 0.001$ compared to geometric mean CFU per milliliter for respective control rats.

compared to the CFU per milliliter at time zero). This inhibition was complement dependent (not detected with heat-inactivated complement) and, as noted above, was not observed in the six strains that were NspA cell surface negative.

Passive protection by anti-rNspA antiserum. The ability of the anti-rNspA antiserum to confer passive protection against bacteremia was evaluated in infant rats subjected to i.p. challenge. Two representative strains were selected for this study, i.e., M986, which was negative for NspA surface accessibility by the flow cytometric assay and resistant to anti-NspA-induced bactericidal activity, and 8047, which was positive for NspA surface-accessible epitopes and susceptible to anti-NspA bactericidal activity. The challenge doses used were similar for the two strains (5.5×10^5 cells of strain M986 and 6.3×10^5 cells of 8047). To maximize the likelihood of observing protection, the bacteria were resuspended in buffer and different dilutions of test or control antisera immediately before the challenge. Table 2 summarizes the results of quantitative bacterial cultures performed on blood specimens obtained 18 h after challenge. A dose of 2 µg per rat of the positive control anticapsular MAb, SEAM 3, was completely protective against both strains. Two dilutions (1:5 and 1:25) of the anti-rNspA antiserum (the highest dilutions tested) protected against bacteremia caused by strain 8047 (positive for NspA surface epitopes). However, neither dilution conferred protection against strain M986 (negative for NspA surface-accessible epitopes).

DISCUSSION

In 1997, Martin et al. described NspA as a potential vaccine candidate for the prevention of invasive *N. meningitidis* disease (36). NspA epitopes appeared to be highly conserved across *N. meningitidis* based on reactivity with a murine anti-NspA MAb, designated Me-1. This MAb cross-reacted with 248 of 250 meningococcal strains in a colony blot ELISA with whole bacterial antigens. This strain collection included representatives of serogroups A, B, C, 29E, W-135, Y, and Z (including 44

serogroup B organisms). The MAb also was bactericidal against isolates with serogroups A, B, or C. In mice, rNspA administered with QuilA elicited serum bactericidal antibody responses against representative strains of serogroups A, B, and C and protected animals from challenge with live *N. meningitidis* group B bacteria. Recently, rNspA absorbed to alum also was reported to elicit serum meningococcal bactericidal antibody responses in rabbits and monkeys (37). Based on these data, an rNspA vaccine is being developed for the prevention of meningococcal disease caused by all serogroups. However, serogroup A and C polysaccharide-protein conjugate vaccines are highly immunogenic in human infants (8, 16, 29, 32) and also induce polysaccharide-specific memory B cells (28, 30). Thus, the greatest utility for an rNspA vaccine would be for prevention of serogroup B disease, since, to date, no other approach to vaccination against serogroup B strains has proven to be safe and broadly effective (18). Therefore, in the present study, we focused on further characterization of NspA in a collection of 17 *N. meningitidis* group B strains that were selected based on ET and/or multilocus sequence typing to be genetically highly diverse (Table 1).

NspA is reported to be accessible at the surface of all intact *N. meningitidis* strains tested (36, 44). However, we found no detectable anti-rNspA antibody binding to the bacterial surface of 6 (35%) of 17 meningococcal B strains as assessed by indirect-fluorescence flow cytometry. The six strains that were negative for anti-rNspA antiserum binding had *nspA* genes that were identical or nearly identical to those of representative strains that were positive for anti-rNspA cell surface binding. Furthermore, based on SDS-PAGE and Western blotting, all six strains that were negative for anti-rNspA antisera binding contained NspA in detergent-insoluble outer membrane preparations. Thus, failure of these strains to express NspA epitopes on the bacterial surface is not a result of absence of the gene, lack of expression of the gene, or polymorphisms.

At present it is unclear why there are strain differences in NspA surface epitopes. One possible clue is our finding that the 6 strains that were negative for surface-reactive NspA produced, on average, greater than threefold more capsular polysaccharide than did the 11 strains that were positive for surface-reactive NspA. Conceivably, the presence of larger amounts of capsule interferes with the ability of the anti-rNspA antibody to bind to NspA epitopes, which, in strains with smaller amounts of capsule, are accessible to antibodies. To gain insight into the possible antigenic sites of NspA and into why NspA surface accessibility might be restricted by the presence of increased amounts of capsular polysaccharide, we have developed a tentative model for the structure and the organization of NspA in the bacterial membrane (Fig. 5).

Based on the pattern of alternating hydrophobic and hydrophilic amino acids, which is characteristic of many β -barrel porin structures (66), we predict that NspA contains eight transmembrane β -strands and four surface-exposed connecting loops. According to Martin et al., the only significant homology between the deduced amino acid sequence of NspA and those of other proteins are weak homologies to the *Neisseria* opacity protein (Opa) family in two small segments (~20 amino acids) near the C-terminal end of the protein (36). However, when we compared the N-terminal and C-terminal halves of NspA separately with sequences in the nonredundant GenBank CDS database (1), we found a high degree of homology (>40% identity and >70% similarity) between NspA and Opa proteins from *N. meningitidis*, *N. gonorrhoeae*, *N. flavus*, *N. sicca*, and *H. influenzae* (data not shown). The Opa proteins are thought to be integral membrane proteins that have eight transmembrane segments and a β -barrel topology in

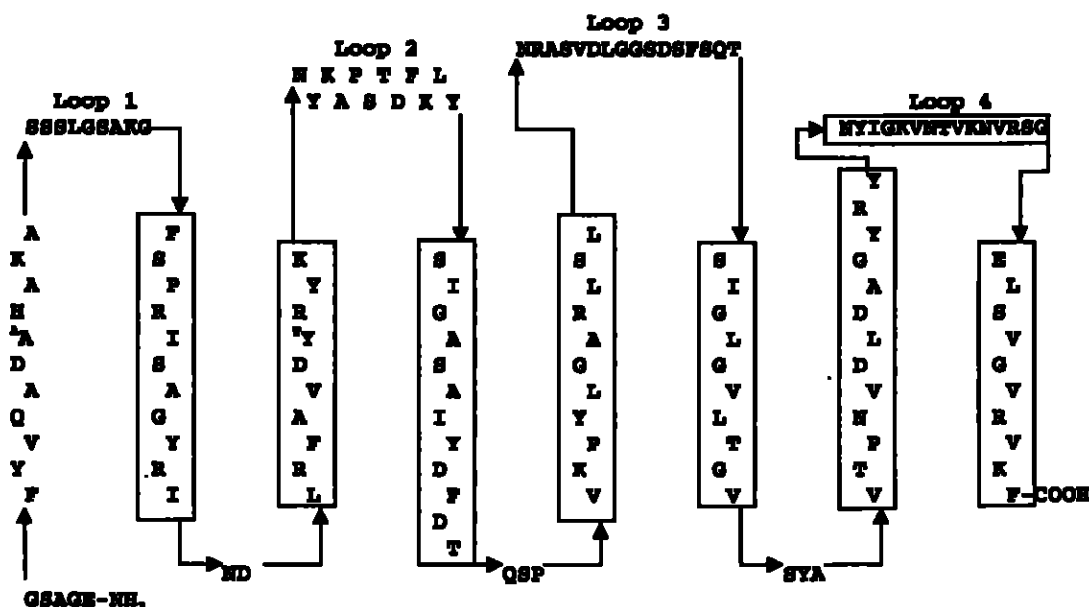


FIG. 5. Secondary-structure prediction for NspA. The boxed areas indicate segments that are >40% identical and >70% similar to encoded amino acid sequences of opacity proteins (Opa) from *N. meningitidis*, *N. gonorrhoeae*, *N. flavus*, *N. sicca*, and *H. influenzae* identified in a BLAST sequence alignment search (1) of the Non-Redundant GenBank CDS. The segments having alternating placement of letters are predicted amphiphilic β -strands. Vertical segments correspond to putative transmembrane segments. Segments at the top of the figure are predicted to be surface-exposed loops, which are labeled loops 1 to 4.

the membrane similar to that of porin (34). The presence of NspA in detergent-insoluble membrane preparations indicates that NspA is located in the outer membrane of meningococci, which would be consistent with the Opa-like membrane topology shown in the model. In addition, the segments of NspA that are most similar to those of the Opa proteins are the putative transmembrane segments and loop 4 indicated by the boxed areas in Fig. 5. Finally, as was found for Opa (46), NspA is a heat-modifiable protein, as shown in Fig. 1C.

The opacity proteins of *Neisseria* may be virulence factors (65) and, under certain circumstances, can elicit protective antibody (50). However, problems with limited antibody accessibility of the opacity proteins in encapsulated bacteria, variability of amino acid sequences in exposed loop segments, and phase variation of protein expression during clinical infection have limited the ability of Opa to elicit protective antibody consistently (34).

In contrast, there appears to be little or no sequence variation in the putative surface-exposed loops of NspA based on the sequence data produced in this study and by others. However, Plante et al. recently reported that a panel of anti-*N. meningitidis* NspA MAbs that reacted with all meningococcal strains tested, reacted with only a limited number of *N. gonorrhoeae* strains, even though the respective amino acid sequences in the two species are 92% identical (44). When the respective NspA sequences of the meningococcal and gonococcal strains were compared (Fig. 4), all of the respective amino acid differences that resulted in changes in hydrophobicity or charge were found to be located in the putative surface-exposed connecting loops (Fig. 5). This finding suggests that the connecting loops in NspA, which are highly conserved in *N. meningitidis*, may be important epitopes for antibodies that bind to native meningococcal NspA. However, the putative surface loops of NspA are relatively small (10 to 16 amino acids) compared to, for example, the highly immunogenic external VR1 and VR2 loops (loop 1 and loop 4, respectively) of

PorA (~10 to 40 amino acids) (61). Although there is some overlap, the NspA loops would appear to be smaller, which might account for lower surface accessibility of NspA epitopes, especially in the presence of abundant capsular polysaccharide.

The full implications of our data with respect to the potential protective efficacy of an rNspA vaccine require additional study. However, our observations suggest that approximately one-third of meningococcal B strains have decreased expression of cell surface NspA epitopes when grown in vitro. Furthermore, strains that are negative for NspA cell surface epitopes are also resistant to anti-NspA-induced complement-mediated bacteriolysis, and for one of the strains tested in the infant-rat model, the anti-NspA antiserum failed to confer protection against bacteremia. In contrast, this antiserum was highly protective against a test strain that was positive for NspA surface-accessible epitopes and that was susceptible to anti-NspA antibody-induced bactericidal activity. Finally, the anti-rNspA-resistant strains also tended to be the same strains that produced the largest amounts of capsular polysaccharide and therefore would be expected to have the greatest virulence. Taken together, these data raise concerns about the ability of a vaccine containing only rNspA to confer broadly protective immunity against meningococcal B disease. An rNspA vaccine, therefore, may need to be supplemented by the inclusion of additional antigens, such as PorA, which is an important target of bactericidal antibody in humans (18). However, before reaching this conclusion, it will be important to investigate the immunogenicity of NspA in humans and to determine whether the addition of other candidate meningococcal antigens to an rNspA vaccine can augment protective immune responses.

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Functional Activity of Anti-Neisserial Surface Protein A Monoclonal Antibodies against Strains of *Neisseria meningitidis* Serogroup B

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Neisserial surface protein A (NspA) is currently being investigated with humans as a candidate vaccine for the prevention of meningococcal disease. Although NspA is highly conserved, the ability of anti-NspA antibodies to bind to or elicit complement-mediated bactericidal activity against diverse *Neisseria meningitidis* serogroup B strains is controversial. To evaluate strain differences in NspA surface accessibility and susceptibility to bactericidal activity, we prepared murine immunoglobulin G2a anti-NspA monoclonal antibodies (MAbs) and evaluated their functional activity against 10 genetically diverse *N. meningitidis* serogroup B strains. By colony Western blot, all 10 strains expressed NspA as detected by one or more MAbs. By flow cytometry, two MAbs were found to bind to the bacterial surface of 6 of the 10 strains. In addition, two strains showed variable NspA surface accessibility for the MAbs despite being uniformly positive for NspA expression by colony Western blotting. Only 4 of the 10 strains were susceptible to anti-NspA complement-mediated bacteriolysis. Passively administered MAb protected infant rats from developing bacteremia after challenge with *N. meningitidis* serogroup B strain 8047 (surface binding positive, susceptible to anti-NspA bacteriolysis), was poorly protective against strain BZ332 (surface binding variable, resistant to bacteriolysis), and did not protect against strain M986 (surface binding negative, resistant to bacteriolysis). Finally, NspA does not appear to be critical for causing bacteremia, as an NspA knockout from strain 8047 was highly virulent in infant rats. Taken together, these findings suggest that an NspA-based vaccine will need to incorporate additional antigens to elicit broad protection against *N. meningitidis* serogroup B.

Neisseria meningitidis serogroup B is an important cause of meningitis and sepsis. Efforts to develop an *N. meningitidis* serogroup B vaccine have been hampered by poor immunogenicity of the polysaccharide capsule, which is cross-reactive with host polysialic acid, and the potential danger of eliciting anticapsular autoantibodies. Alternative approaches using noncapsular antigens are being investigated (8). However, the ability of most noncapsular antigens to elicit broadly protective responses is limited by antigenic heterogeneity (15, 17, 20, 21, 24), and/or variable expression of the surface-exposed epitopes (3, 11, 22). One possible exception is *Neisserial surface protein A (NspA)*, first described in 1997 by Martin and colleagues (13). NspA is highly conserved (4, 12, 13, 16, 19; D. Martin, C. R. Rioux, A. Villeneuve, J. Hamel, and B. R. Brodeur, Abstr. 11th Int. Pathog. *Neisseria* Conf., p. 198, 1998), appears to be expressed in all *N. meningitidis* serogroup B strains tested (13, 16), and elicits protective antibody responses in mice against strains of *N. meningitidis* serogroups A, B, and C (13). Murine monoclonal antibodies (MAbs) prepared against rNspA are also reported to be broadly cross-reactive and protective against *N. meningitidis* serogroup B strains (4, 12, 13).

Despite gene conservation and expression of NspA by all meningococcal strains tested to date, our laboratory noted differences among *N. meningitidis* serogroup B strains in bacterial surface binding, as determined by flow cytometry and complement-mediated bactericidal activity of mouse polyclonal anti-rNspA antisera (16). Binding and bactericidal activity were lowest for strains producing the greatest amount of

capsular polysaccharide (16). While other factors may be important, these data suggest that the capsule can limit the accessibility of NspA surface epitopes and/or block complement-mediated bacteriolysis evoked by anti-NspA antibody binding. Therefore, an NspA-based vaccine might have only limited ability to elicit protective immunity against highly encapsulated strains, the very strains expected to have the greatest virulence (7, 10, 14, 23).

In contrast, Martin et al. (11th Int. Pathog. *Neisseria* Conf., 1998), using anti-NspA MAbs, reported that NspA was accessible on the cell surface and elicited complement-mediated bacteriolysis of nearly all meningococcal strains tested and that the MAbs could passively protect mice challenged with different strains (4).

To resolve the discrepancies among these observations, we prepared a panel of murine MAbs to rNspA. Herein, we report the ability of these MAbs to bind to the bacterial surface, to elicit complement-mediated bactericidal activity, and to passively protect infant rats challenged with *N. meningitidis* serogroup B strains.

MATERIALS AND METHODS

Bacterial strains. The 10 *N. meningitidis* serogroup B strains chosen for this study (Table 1) were selected to be genetically diverse and representative of the 17 strains examined in an earlier study (16). The *N. meningitidis* serogroup B collection included five strains previously considered to be NspA surface positive using polyclonal anti-rNspA antisera and five strains considered NspA surface negative. The five surface-positive strains included three that were susceptible to anti-rNspA complement-mediated bacteriolysis and two that were resistant. All five surface-negative strains were also resistant to bacteriolysis with polyclonal anti-rNspA antisera. The 10 strains were isolated over a period of 25 years from patients residing in different countries. Based on electrophoretic typing (ET), the strains represent a broad range of genetic diversity for *N. meningitidis* serogroup B strains causing disease (5, 18).

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TABLE 1. *N. meningitidis* serogroup B strains

Strain	Country of origin	Year isolated	Reference	Serologic classification	ET complex ^d	NpA sequence ^e	Previously reported anti-NpA reactivity ^a	
							Surface binding	Bactericidal activity
8047	U.S.	1978	Zollinger, CHORI ^b	2b:P1.5,2	ND	yes	+	+
CU385	Cuba	1980	Sacchi et al.	4,7:P1.19,15	5	yes	+	+
BZ198	The Netherlands	1986	Caugant et al.	NT:P.NST ^c	154	no	+	+
S3446	U.S.	1973	Sacchi et al.	19,14:P1.23,14	8	no	+	— (static)
1000	USSR	1989	CHORI	NT:P1.5	61	no	+	—
BZ232	The Netherlands	1964	Caugant et al.	NT:P1.2	76	yes	—	—
NG3/88	Norway	1988	Caugant et al.	8:P1.1	A4 complex	yes	—	—
NGP165	Norway	1974	CHORI	NT:P1.2	37	yes	—	—
MCS8	United Kingdom	1985	McGuinness et al.	15:P1.7,16	5	yes	—	—
M986	U.S.	1963	Sacchi et al.	2a:P1.5,2	165	yes	—	—

^a Previously reported results from Moe et al. using murine polyclonal antisera (16). Surface binding was determined by flow cytometry. Static, strains that were inhibited but not killed in the assay (≥ 50 but $< 100\%$ survival at 60 min).

^b CHORI, determined by our laboratory.

^c NT, nonserotypeable. NST, nonserosubtypeable.

^d Determined by electrophoretic typing (ET) (6); ND, not determined.

^e Previously reported (16).

Mutants of strains 8047 and BZ198 (8047ANpA and BZ198ANpA, respectively), in which the *nspA* gene was inactivated, were prepared by transforming the parent strain with the plasmid pBSUDNpAERM (gift from J. Abu-Bobie, Chiron Corp., Siena, Italy). The plasmid contains a truncated *nspA* gene and the *ermC* gene (erythromycin resistance). The bacteria were transformed by selecting a few colonies grown overnight on chocolate agar plates (Remel, Lenexa, Kans.) and mixing them with 20 μ l of phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and 1 μ g of plasmid DNA. The mixture was spotted onto a chocolate agar plate, incubated for 6 h at 37°C, 5% CO₂, and then diluted in PBS-BSA and spread on chocolate agar plates containing 7 μ g of erythromycin per ml. The presence of the disrupted *nspA* gene in the genome of both strains was confirmed by PCR using the following primers: 5'-ACAGCAG QATCCTTTAACGGATTTC-3' and 5'-GTGATGAAGCTTTGACATTTC-3'. Lack of NpA expression was confirmed by whole-cell enzyme-linked immunosorbent assay (ELISA) as described below and Western blots of outer membrane proteins prepared from the knockout strains and resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels as described previously (16).

Preparation of MAbs. Female CD1 mice (Charles River, Hollister, Calif.) were vaccinated with microvesicles prepared from a transformed *Escherichia coli* strain that expressed recombinant NpA from a multicopy plasmid containing the *nspA* gene under the control of its own promoter (plasmid pGMS1.0) (16). The mice were given three 100- μ l injections, each separated by 3 weeks, containing 10 μ g of protein and 10 μ g of CpG oligonucleotides (5'-TCCATGACG TTCTGACGTT-3'). The first two doses were given subcutaneously together with aluminum phosphate (0.5%, w/vol), and the final dose was given without adjuvant and administered intraperitoneally (i.p.). Three days later, the animals were sacrificed and their spleen cells were fused with myeloma cells (P3X63-Ag8.653) at a ratio of 1 spleen cell to 1.7 myeloma cell. After two weeks of incubation in hypoxanthine-aminopterin-thymidine selective medium, hybridoma supernatants were screened for antibody binding activity by ELISA using encapsulated *N. meningitidis* serogroup B strains BZ198 as the target antigen (see description below). Specificity of antibody binding to NpA was demonstrated by lack of binding in a replicate ELISA performed with BZ198ANpA. Hybridomas secreting NpA-specific antibody were cloned by limiting dilution and then expanded and frozen for subsequent use in tissue culture.

Antibodies from four cell lines were characterized in detail. The subclasses of the MAbs were determined using an antibody capture ELISA and alkaline phosphatase-conjugated polyclonal antibody specific for each of the mouse immunoglobulin G (IgG) subclasses, IgM, IgA, and κ and λ light chains (Southern Biotechnology Associates, Inc., Birmingham, Ala.) (9). The MAbs produced by the hybridoma clones were harvested from tissue culture media by ammonium sulfate precipitation (55%, w/vol) and, after exhaustive dialysis in PBS buffer, purified by affinity chromatography using a Poros Q/M column (Applied Biosystems, Foster City, Calif.). The concentration of the purified MAb was determined spectrophotometrically assuming a 1-mg/ml antibody solution having an absorbance of 1.35 at 280 nm.

Binding of antisera to the surface of live encapsulated meningococci. The ability of the MAbs to bind to the surfaces of live *N. meningitidis* serogroup B strains was determined using a flow cytometric detection of indirect fluorescence assay, performed as described previously (16). Positive-control antibodies included meningococcal-specific serotyping or subtyping MAbs (MN14C11.6, MN16C13P4, Rijksinstituut Voor Volksgezondheid en Milieu, Bilthoven, The Netherlands) and SEAM 12, an antipolysaccharide MAb that is specific for encapsulated group B strains (9). The negative control consisted of a mouse IgG MAb (VIG10) of irrelevant specificity.

Complement-dependent bactericidal antibody activity. (I) Low inoculum assay. After overnight growth on chocolate agar, 5 to 10 colonies were inoculated into Mueller-Hinton broth (starting optical density at 620 nm [OD₆₂₀] of ~0.1) and the test organism was grown for approximately 2.5 h to an OD₆₂₀ of ~0.6 (early log phase). One of the test strains, CU385, was grown in an identical manner except that glucose (0.25%, w/vol) was added to the broth. In the absence of glucose in the broth, the resulting bacteria from this strain did not show an increase in CFU/ml during the bactericidal assay (see below). To prepare the bacteria for the assay, the cells were washed once in Gey's balanced salt solution (Gibco BRL, Rockville, Md.) containing 1% (w/vol) BSA (Gey's-BSA). After an appropriate dilution, 12 μ l containing approximately 300 to 400 CFU was added to a final reaction volume of 60 μ l, consisting of 20% (vol/vol) complement and serial twofold dilutions of MAbs in Gey's-BSA buffer. The complement source was human serum from a healthy adult (MAS) with no detectable antipolysaccharide antibody to group B polysaccharide as tested by ELISA and no detectable intrinsic bactericidal activity against the test strain when the serum was tested at a final concentration of 20 or 40%. After incubation at 37°C for 60 min, 20 μ l of the reaction mixture was transferred to a Mueller-Hinton plate as previously described (16). Serum bactericidal titers (BC₅₀) were defined as the antibody concentration resulting in a 50% decrease in CFU per ml after 60 min of incubation of bacteria in the reaction mixture, compared to the control CFU per ml at time zero. Typically, bacteria incubated with the negative-control antibody and complement showed a 150 to 200% increase in CFU/ml during the 60 min of incubation.

(II) High-inoculum bactericidal assay. This assay was adapted from that previously described by Amir et al. for measuring bactericidal activity against *Haemophilus influenzae* type b (2). The assay is essentially the same as the low-inoculum assay except that the final reaction mixture (120 μ l to 1 ml) contained a fixed concentration (100 μ g/ml) of anti-NpA MAb AL12 diluted in Gey's-BSA buffer, 20% (vol/vol) human serum as a complement source and ~10⁸ CFU/ml log-phase meningococcal cells diluted in Gey's-BSA. Controls included the test organism incubated in the absence of antibody (complement alone) or MAb in the presence of heat-inactivated (56°C for 30 min) complement. The reaction vials were incubated at 37°C on a Clay Rotator orbital mixer (Fisher Scientific, Pittsburgh, Pa.) for 60 min, and serial dilutions were plated onto chocolate agar plates. The results were expressed as the log decrease in meningococcal cells as compared to that of control bacteria incubated with MAb and inactivated complement.

Expression of NspA by colony blot assay. In order to determine whether particular bacterial colonies were expressing NspA, the colonies were transferred from the surface of chocolate agar plates onto nitrocellulose filters (Bio-Rad, Richmond, Calif.) by blotting. The filters were washed once with PBS buffer containing 0.1% (wt/vol) Tween 20 (Sigma, St. Louis, Mo.) and 0.1% (wt/vol) sodium azide (wash buffer) and then blocked with wash buffer containing 1% (wt/vol) nonfat dry milk (blocking buffer). NspA was detected by adding a solution of anti-NspA MAb AL12 (~0.1 µg/ml) in blocking buffer, incubating at ambient temperature for 2 h, and then washing the filters with wash buffer. Bound antibody was detected by adding rabbit anti-mouse IgG-, IgA-, and IgM-alkaline phosphatase conjugated polyclonal antibody (Zymed, South San Francisco, Calif.) diluted in wash buffer containing 1% (wt/vol) BSA. After incubation for 1 h at ambient temperature, the filters were washed as described above, and Sigma Fast BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium) substrate was added.

ELISA. The whole-cell ELISA assay was performed as described by Abdullahi and Poolman (1). Briefly, bacterial cells grown overnight at 37°C in 5% CO₂ on chocolate agar plates were resuspended in sterile PBS buffer and then inactivated by heating to 56°C in a water bath for 30 min. The suspension was adjusted to an OD₆₀₀ of 0.1, and 100 µl of the suspension was added to wells of flat-bottom 96-well microtiter plates (Nalge Nunc International, Rochester, N.Y.). The liquid in the wells was allowed to evaporate at ambient temperature in a fume hood. Plates containing the dried bacteria were stored at ambient temperature until use. Before adding antibodies, the plates were washed once with wash buffer and blocked by adding blocking buffer and incubating at 37°C for 1 h. After removal of the blocking buffer, test antibodies diluted in blocking buffer were added to the wells and incubated at 4°C overnight. The plates were washed five times with wash buffer followed by the addition of rabbit anti-mouse IgG-, IgA-, and IgM-alkaline phosphatase conjugated polyclonal antibody (Zymed) diluted in wash buffer containing 1% (wt/vol) BSA. After 1 h of incubation at ambient temperature, the plates were washed five times with wash buffer and developed with *p*-nitro phenylphosphate substrate (1 mg/ml; Sigma) in 1 M diethanolamine, pH 9.8, containing 0.5 mM magnesium chloride. The OD₄₀₅ was measured using a microtiter plate reader.

The specificity of the anti-NspA MAbs for NspA was determined using the following solid-phase antigens: microvesicles prepared from *E. coli* expressing rNspA and microvesicles prepared from *E. coli* transformed with the parent plasmid lacking the *nspA* gene, lauroyl sarcosinate-insoluble outer membrane proteins prepared from *N. meningitidis* serogroup B strains BZ198 and BZ198ΔNspA, and recombinant HisTag NspA purified by Ni-NTA Sepharose metal affinity chromatography. Methods used for preparing the antigens were the same as described previously (16). The antigen preparations were diluted in PBS buffer (10 to 100 µg of total protein per ml), added to the wells of microtiter plates, and allowed to bind at 4°C overnight. After washing the plates with wash buffer, the plates were blocked with PBS buffer containing 1% (wt/vol) BSA. The anti-NspA and control MAbs were diluted in wash buffer containing 1% (wt/vol) BSA or, for HisTag NspA, PBS buffer containing 1% (wt/vol) BSA and 0.3% (wt/vol) Emulgen BB (Calbiochem, La Jolla, Calif.). After overnight incubation at 4°C, the plates were washed, incubated with secondary antibody, and developed as described above.

Animal protection. The ability of the anti-rNspA antiserum to confer passive protection against *N. meningitidis* serogroup B bacteremia was tested in infant rats (16). In brief, 5- to 8-day old pups from litters of outbred Wistar rats (Charles River, Raleigh, N.C.) were randomly redistributed to the nursing mothers. Three strains, M986, BZ232, and 8047, were tested. Each strain had been serially passaged three times in infant rats. After the third passage, the blood cultures from the rats were grown overnight on chocolate agar. The bacteria were suspended in sterile skim milk and stored frozen at -80°C. On the day before challenge, freshly thawed bacteria were inoculated onto chocolate agar and grown overnight at 37°C in 5% CO₂. On the morning of the challenge, colonies were picked, inoculated into a broth culture, and grown and prepared as described above for the bactericidal assay. In two experiments using strains 8047 and BZ232, the animals were pretreated i.p. with 100 µl of different concentrations of test or control MAbs or buffer alone. Two hours later, the animals were challenged i.p. with approximately 10³ to 10⁶ CFU of *N. meningitidis* serogroup B bacteria. To enhance the sensitivity of the assay, in the third experiment with strain M986, the bacteria were suspended in different concentrations of test or control MAbs immediately before the challenge and 100 µl of the mixture was administered i.p. Heparinized blood specimens were obtained by heart puncture 18 h after the bacterial challenge. In one experiment with strain M986, blood samples were also obtained 6 h after challenge. Aliquots of 1, 10, and 100 µl of blood were plated onto chocolate agar. The number of CFU per milliliter of

blood was determined after overnight incubation of the plates at 37°C in 5% CO₂.

RESULTS

Production of anti-NspA MAbs. Polyclonal sera from individual mice immunized with *E. coli* microvesicles containing rNspA were evaluated for antibody binding by ELISA and complement-mediated bactericidal activity. As the target antigen, the ELISA included encapsulated *N. meningitidis* serogroup B cells from strain BZ198 or the same strain in which the NspA gene had been deleted (BZ198ΔNspA). With anti-rNspA antisera, strain BZ198 gave the best binding in the whole-cell ELISA compared to that of the other strains. The bactericidal assay included two test *N. meningitidis* serogroup B strains, CU385 and 1000 (Table 1). Based on the results of the ELISA and bactericidal assays, a high-responder mouse was selected for the fusion. From this fusion, we obtained five hybridoma cell lines producing antibodies that were positive by ELISA for binding to strain BZ198 but were negative for binding with the NspA knockout strain, BZ198ΔNspA. Each hybridoma cell line was subsequently cloned and grown on a larger scale in tissue culture to produce larger amounts of antibody for further characterization. The MAbs expressed by each of the five clones were designated AL4, AL5, AL8, AL11, and AL12, and were IgG2a(κ). Because of low binding and functional activity of AL8, the results reported below are limited to the remaining four MAbs.

By ELISA, the four MAbs bound to microvesicles prepared from the *E. coli* strain containing recombinant NspA, which had been used to immunize the mouse for preparation of the hybridomas. In contrast, only background binding was observed when the MAbs were tested at 10-fold higher concentrations against microvesicles prepared from the same *E. coli* strain transformed with the plasmid without the *nspA* gene (Fig. 1A). Similarly, the four MAbs bound to lauroyl sarcosinate-insoluble outer membrane proteins prepared from *N. meningitidis* serogroup B strain BZ198 but were negative when tested at 10-fold higher concentrations with outer membrane proteins prepared from strain BZ198ΔNspA, which does not express NspA (Fig. 1B). Finally, the four MAbs bound by ELISA to affinity-purified HisTag-NspA. In contrast, a negative-control MAb, VIG10, with an irrelevant specificity, showed only background binding when tested at 10-fold higher concentrations (Fig. 1C). The above results show that the four MAbs are specific for binding to NspA. Note that much higher concentrations of the MAbs were required for binding to HisTag-NspA than to NspA present in the *E. coli* or *Neisseria* membrane preparations. This result likely reflects the loss of conformational epitopes in the HisTag-NspA eluted from the affinity column in the presence of 8 M urea.

Figure 2 shows binding of the MAbs to heat-killed, whole bacterial cells from strain BZ198 as measured by ELISA. All four MAbs showed similar dose-response binding. All four MAbs showed only background binding (OD₄₀₅ < 0.05) when bacterial cells from strain BZ198ΔNspA were employed as the target instead of the respective BZ198 wild-type strain.

Anti-rNspA MAb cell surface binding as determined by indirect fluorescence flow cytometry. The four purified MAbs were tested by flow cytometry for their ability to bind to live

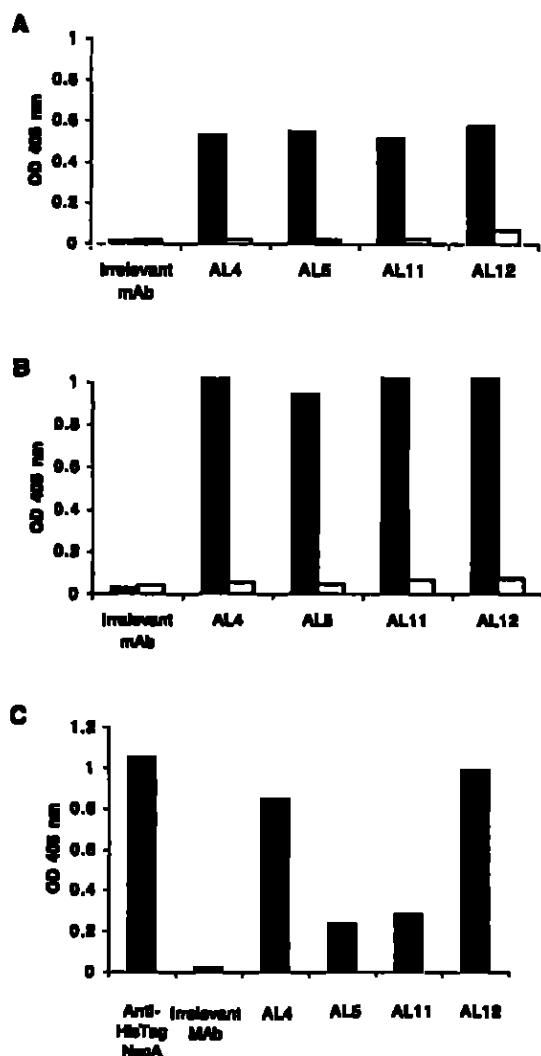


FIG. 1. Binding of the anti-NspA MAbs by ELISA. (A) Binding to microvesicles prepared from an *E. coli* strain expressing recombinant NspA (closed bars) or from the same *E. coli* strain transformed with the plasmid without the *nspA* gene (open bars). Data for the microvesicles containing rNspA were obtained at an antibody concentration of 60 ng/ml, whereas the negative control microvesicles were tested at 600 ng/ml. (B) Binding to lauroyl sarcosinate-insoluble outer membranes prepared from *N. meningitidis* serogroup B strain BZ198 (closed bars) or BZ198ΔNspA (open bars), in which the gene encoding NspA had been inactivated. The antibody concentrations were the same as those for panel A. (C) Binding to Ni-NTA Sepharose metal affinity-purified HisTag-NspA. The anti-NspA MAbs were tested at 5 μg/ml, the irrelevant MAb was tested at 50 μg/ml, and the polyclonal anti-HisTag NspA antisera were tested at a dilution of 1:108,000.

cells from various *N. meningitidis* serogroup B strains. Representative data for three strains, BZ198, 8047, and M986, are shown in Fig. 3. The anticapsular MAb SEAM 12 bound to all three strains, whereas an irrelevant MAb, VIG10, showed only background binding. With strain BZ198, the four MAbs were all positive for binding when tested at 6 μg/ml. In contrast, with strain M986, all four MAbs were negative for binding when tested at 60 μg/ml. Note that with this strain, a positive-control

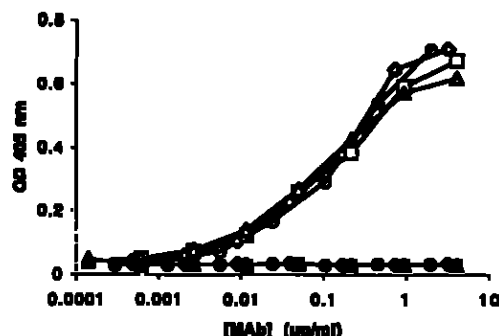


FIG. 2. Whole-bacterial-cell ELISA comparing binding activities of the anti-NspA MAbs AL4 (○), AL5 (□), AL11 (Δ), and AL12 (○) to *N. meningitidis* serogroup B strain BZ198. The respective filled symbols show the results from a comparable experiment with strain BZ198ΔNspA as the solid-phase antigen.

anti-PorA P1.2 MAb (MN16C13F4) bound strongly when tested at a dilution of 1:20,000 (Fig. 3). With strain 8047, AL12 was positive at 10 and 60 μg/ml (data only shown for the higher dose), and AL4 was positive only when tested at the higher antibody concentration (60 μg/ml). AL5 and AL11 were negative at the highest concentrations tested (60 μg/ml). Thus, based on the flow cytometry results, the four clones appear to be different. Also, MAbs AL4 and AL12 showed the best binding of the four MAbs when tested with strain 8047.

Variation in anti-NspA surface binding of AL4 and AL12 was apparent for two strains, BZ232 and MCS8. Representative results with strain MCS8 are shown in Fig. 4 for three separate subcultures of MCS8 tested in the same experiment. Isolate 1 was strongly positive for surface binding with AL12 at 10 μg/ml, the lowest concentration tested. In contrast, isolate 3 showed much lower binding even when tested at 60 μg/ml. Also, there appeared to be a mixed population of positive and negative binding. Binding of isolate 2 appeared to be homogeneous but less than isolate 1 and more than isolate 3. The binding results with AL4 were similar (data not shown). The control anti-PorA (P1.7) and anticapsular MAbs showed comparable respective results of binding to bacteria from all three subcultures. Clonal bacteria obtained from positive and negative subcultures were positive or negative irrespective of whether the parent subculture was positive or negative (data not shown).

The variable binding of AL4 and AL12 to bacteria from different strain MCS8 cultures did not result from failure to express NspA. As shown in Fig. 5B, all bacterial colonies tested from subculture 3 with negative or mixed surface binding with AL4 and AL12 by flow cytometry were positive for NspA expression when tested by colony Western blotting using AL12 as the detecting antibody. For comparison, representative positive and negative colonies from a mixture of strains 8047 and 8047ΔNspA are shown in Fig. 5A.

Table 2 summarizes the results from flow experiments measuring the ability of the different MAbs to bind to the surfaces of live bacteria from each of the 10 strains. All five strains that had been positive for binding with the polyclonal antiserum were positive for binding with MAbs AL4 and AL12. One strain, NQ3/88; that had been negative for binding with poly-

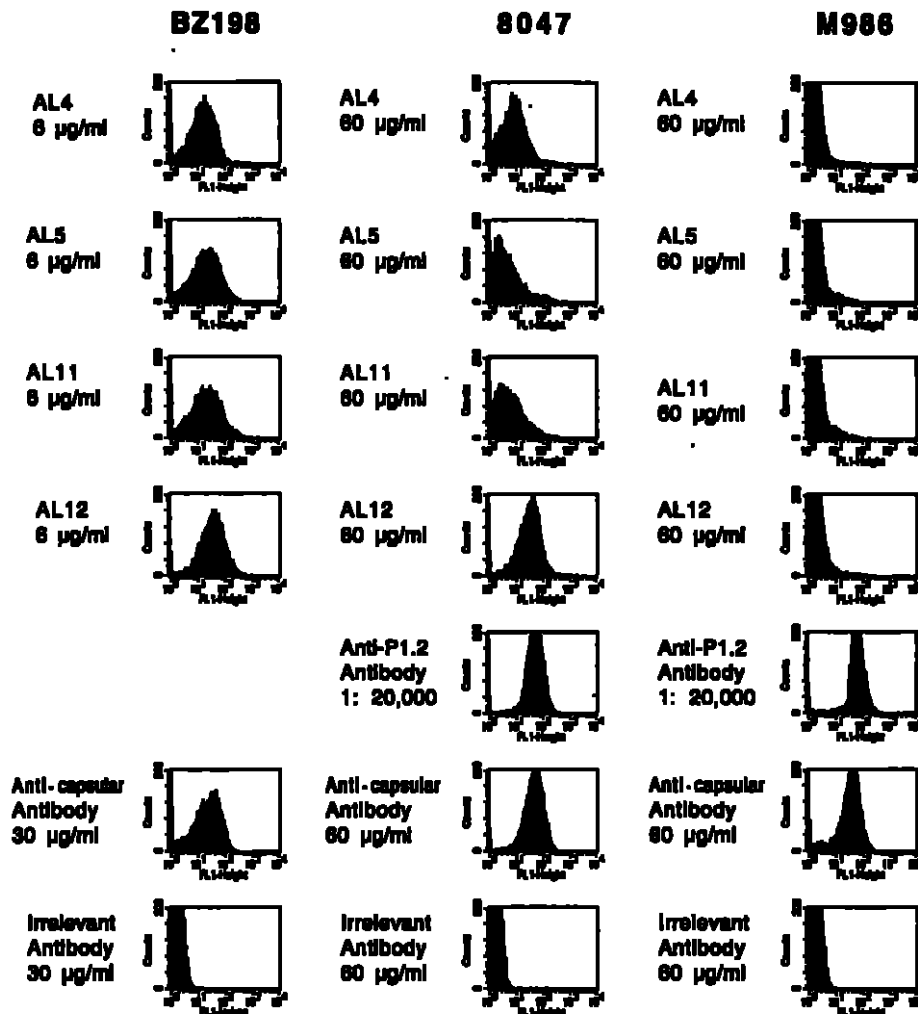


FIG. 3. Binding of anti-rNspA and control MABs to live encapsulated *N. meningitidis* serogroup B strains BZ198, 8047, and M986 as determined by indirect-fluorescence flow cytometry. The anti-NspA MABs include AL4, AL5, AL11, and AL12. The control MABs include a murine MAB with an irrelevant specificity (VIG10), an anticapsular-specific murine MAB (SEAM 12), and, for strains 8047 and M986, an *N. meningitidis* subtype MAB anti-PorA P1.2 (Rijksinstituut Voor Volksgezondheid en Milieu, Bilthoven, The Netherlands). A subtyping anti-PorA MAB was not available for strain BZ198.

clonal anti-NspA antibody in our previous study, was positive for surface binding with these two MABs (compare Tables 1 and 2). Two strains that had been negative with the polyclonal antisera in our previous study (MC58 and BZ232) showed variable binding with the MABs, and two other strains (NGP165 and M986) were negative with both the polyclonal and monoclonal anti-NspA antibodies.

Bactericidal activity. The ability of the anti-rNspA MABs to elicit complement-mediated bacteriolysis of the 10 strains in the low inoculum is summarized in Table 2. MAB AL4, which was positive for surface binding by flow cytometry for six strains, was bactericidal only against strain BZ198. MAB AL12, which was positive for binding by the flow assay for the same six strains as AL4, was bactericidal against four strains. However, for two of these four strains, the antibody concentrations of AL12 required for eliciting bacteriolysis were high (32 and 110 $\mu\text{g/ml}$). The remaining two MABs tested, AL5 and AL11,

were bactericidal only against strains 8047 and BZ198 (AL5) and 8047, BZ198, and S3446 (AL11).

Figure 6 summarizes the bactericidal activity of AL12 when tested in the high inoculum assay in which a fixed concentration of antibody (100 $\mu\text{g/ml}$) is incubated with $\sim 10^8$ CFU/ml of log-phase meningococcal cells and 20% complement. The five strains tested in this assay included one strain that was susceptible to bacteriolysis in the low-inoculum assay (8047), two strains that were moderately resistant (S3346 and 1000), and two strains that were resistant (CU385 and NG3/88). By flow cytometry, all five test strains showed strong binding of AL12 to the bacterial surface. The three strains with $\text{BC}_{50} > 100$ $\mu\text{g/ml}$ in the low-inoculum assay were poorly killed when retested under the high inoculum conditions (no killing of NG3/88, and 0.8 and 1.2 $\log_{10}$ killing of CU385 and 1000, respectively). In contrast, the two strains with BC_{50} of 8 and 32 $\mu\text{g/ml}$ in the low-inoculum assay were efficiently killed under the

MC58

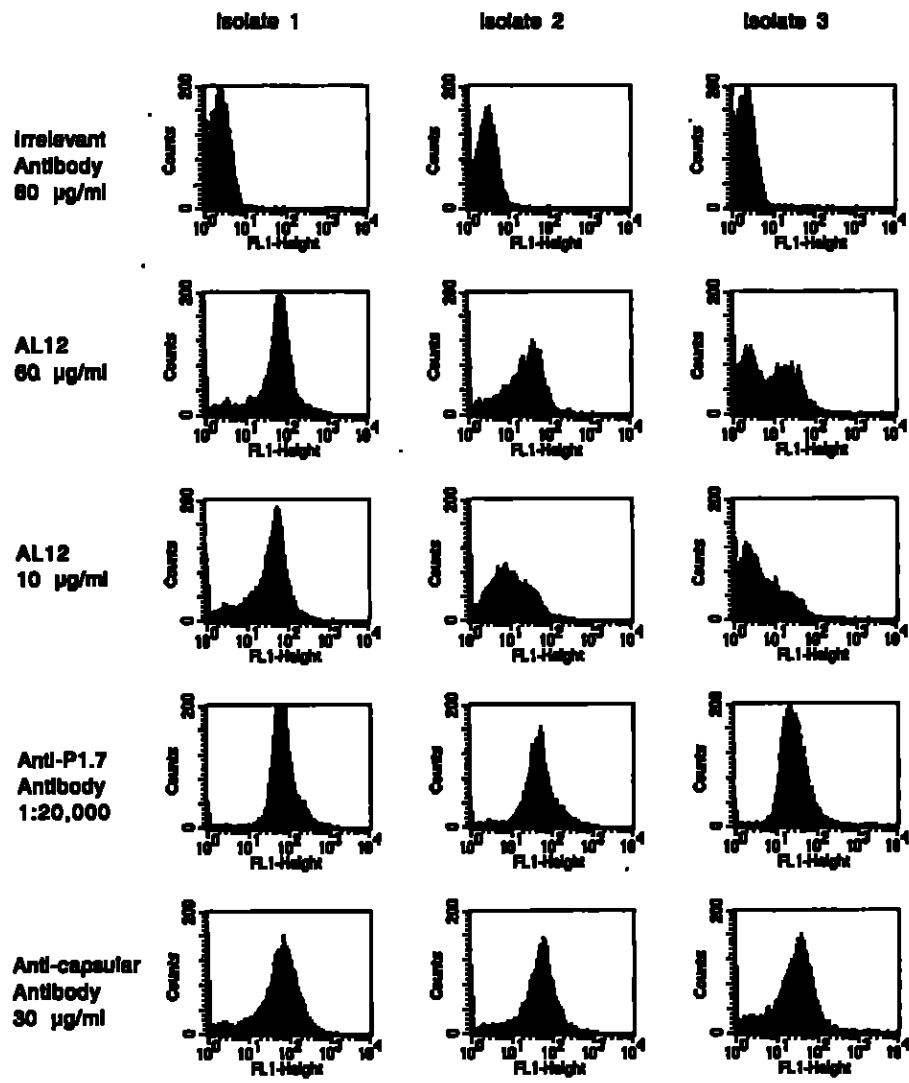


FIG. 4. Binding of anti-NspA MAb AL12 to bacteria grown from different subcultures of strain MC58 as determined by indirect-fluorescence flow cytometry. The murine control MAbs include a MAb of irrelevant specificity, an anti-PorA P1.7 MAb, and an anticapsular MAb.

conditions of the high-inoculum assay (4.4 log₁₀ killing of 8047 and 4.9 log killing of S3446).

The results of the high-inoculum assay provide further support for the functional activity of the anti-NspA antibody against some but not all strains. Since even among susceptible strains not all bacteria are killed under the conditions of this assay, we were also able to examine large numbers of surviving colonies for NspA expression by colony Western blotting. Bacteria surviving after complement-mediated killing with AL12 in the high-inoculum assay were tested for NspA expression by blotting ~1,000 CFU on nitrocellulose filters and probing for NspA expression by Western blotting using AL12 as the primary antibody. All surviving colonies appeared to be strongly positive. Furthermore, repeated selection (two times) of CFU

surviving in the high-inoculum assay with strain S3446 (4.9 and 5.7 log₁₀ killed per selection) did not result in the identification of NspA-negative CFU. As a control for determining whether NspA-negative CFU could be detected in a background of NspA-positive CFU, blotting was performed on a mixture (100:1) of 8047 wild type and 8047ΔNspA. After incubation with AL12 and complement, approximately 90% of the surviving CFU examined were negative for NspA expression. Figure 5A shows an example of a Western blot of colonies from a plate containing a mixture of strains 8047 and 8047ΔNspA probed with AL12.

In the high-inoculum bactericidal assay of strains 8047 and S3446, we observed 4- to 5-log₁₀ decreases in CFU after 60 min of incubation with the MAb and complement. Given the uni-

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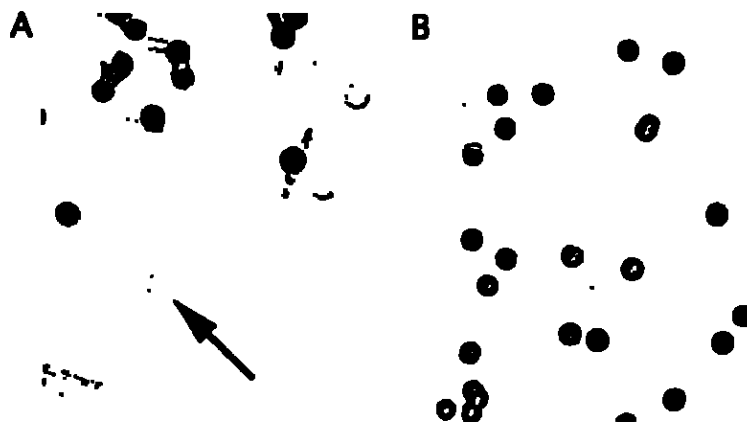


FIG. 5. Colony Western blots using anti-NspA MAb AL12 as the detecting antibody. (A) Mixture of strains 8047 and 8047ΔNspA. The arrow indicates an example of a strain 8047ΔNspA colony. (B) Strain MC58 CFU from the flow experiment depicted in Fig. 3 in which the strain was negative for AL4 and AL12 binding (isolate 3). All colonies tested in the Western blot were positive for AL12 binding.

form expression of NspA among $>1,000$ CFU surviving after the bactericidal assay, we can estimate that the occurrence of an NspA-negative CFU to be <1 in 10^7 to 10^8 bacteria. By comparison, when similar selection experiments were performed with strain 8047 using the anti-PorA P1.2 MAb, CFUs negative for P1.2 were obtained at a frequency of approximately 1 in 10^8 bacteria. Finally, for strains CU385, 1000, and NG3/88, which were moderately resistant to anti-NspA-induced bacteriolysis, we also probed approximately 100 to 1,000 CFU after incubation with AL12 and complement. The CFUs tested from all of the strains showed uniform strong expression of NspA. Thus, resistance of these strains to anti-NspA bacteriolysis was not a result of failure to express NspA or the inability of the MAb to bind to the NspA epitope.

Passive protection by anti-rNspA MAb AL12. The ability of anti-rNspA MAb AL12, the most active MAb in the flow and bactericidal assays, to confer passive protection against meningococcal bacteremia was assessed in an infant rat model. Three experiments were performed (Table 3). In experiment 1, the

animals were pretreated with test or control MABs at time zero and challenged 2 h later with 2.3×10^3 CFU of strain 8047 (positive with AL12 in the flow assay at $10 \mu\text{g/ml}$ and susceptible to bacteriolysis with a BC_{50} of $8 \mu\text{g/ml}$). In experiment 2, the protocol was similar but the animals were challenged with 15×10^3 CFU of strain BZ232 (variable binding in the flow assay and resistant to bacteriolysis). In experiment 3, the animals were challenged with 2×10^3 CFU of strain M986 (negative for binding by the flow assay and resistant to bacteriolysis). Also, in order to maximize the likelihood of observing protection against strain M986 in experiment 3, the bacteria were premixed with the anti-rNspA or control MABs immediately prior to the i.p. challenge. In all three experiments, the positive-control anticapsular MAB (SEAM 3) was protective and there was no protection observed in the animals treated with the irrelevant negative control MAB as determined by comparing the levels of bacteremia in the respective animals treated with PBS-BSA ($P > 0.2$ by t test of the respective geometric mean CFU/ml of blood).

TABLE 2. Summary of anti-NspA MAB surface reactivity for genetically diverse *N. meningitidis* serogroup B strains

Strain	Surface reactivity (flow) of anti-NspA MABs and/or complement-mediated BCA ^a							
	AL4		AL5		AL11		AL12	
	Flow ^b	BCA	Flow	BCA	Flow	BCA	Flow	BCA
8047	+	>60	—	45	—	15	++	8
CU385	++	>120	+	>120	+	>120	++	>120
BZ198	++	26	++	5	++	4	++	5
S3446	++	>60	++	>60	++	60	++	32
1000	+	>120	—	>120	—	>120	++	110
BZ232	variable	>120	—	>120	—	>120	variable	>120
NG3/88	++	>120	+	>120	++	>120	++	>120
NGP165	—	>120	—	>120	—	>120	—	>120
MC58	variable	>120	variable	>120	variable	>120	variable	>120
M986	—	>120	—	>120	—	>120	—	>120
BZ198ΔNspA	—	>60	—	>60	—	>60	—	>60

^a BCA, bactericidal activity (concentration of MAB that when incubated for 60 min with bacterial cells and 20% human complement yielded a 50% decrease in CFU per ml compared to that at time zero).

^b Surface binding was measured by flow cytometry. With one exception, all strains were tested at MAB concentrations of 10 and 60 $\mu\text{g/ml}$. The exception, strain BZ198, was tested at 6 and 60 $\mu\text{g/ml}$. Results were scored as follows: —, less than 50% of total fluorescent events have intensity above background when tested at 60 μg of MAB per ml; +, $\geq 50\%$ of total fluorescent events have intensity above background at 60 $\mu\text{g/ml}$; ++, $\geq 50\%$ of total fluorescent events have intensity above background at 6 or 10 $\mu\text{g/ml}$; variable, subcultures ranged from — to ++ (see text).

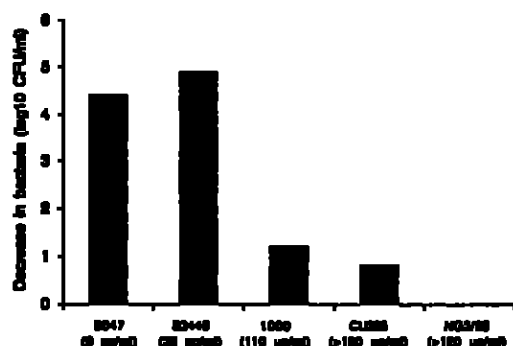


FIG. 6. High-inoculum bactericidal assay. The decrease ($\log_{10}$) in the number of bacteria resulting from bacteriolysis in the presence of complement and anti-NspA MAb AL12, compared to the number of control bacteria incubated with antibody alone or antibody with heat-inactivated complement, is shown. The numbers in parentheses indicate the BC_{50} concentrations of AL12 for each strain measured in the low-inoculum bactericidal assay.

In experiment 1, none of the animals pretreated with anti-NspA MAb AL12 (50 μ g per rat) and challenged by strain 8047 developed bacteremia. At a MAb dose of 5 μ g per rat, four of the seven animals had bacteremia but the geometric mean CFU/ml of the treated animals was less than 1/50 of that of control animals pretreated with the irrelevant MAb or PBS-BSA ($P < 0.005$). In experiment 2, four of five animals pretreated with 50 μ g of AL12 per rat and four of five animals pretreated with 10 μ g per rat had bacteremia 18 h after challenge by strain BZ232. However, at both antibody doses, the respective geometric mean CFU/ml of blood of the treated animals was lower than that of control animals pretreated with an irrelevant MAb or PBS-BSA ($P < 0.01$). In experiment 3,

TABLE 4. *N. meningitidis* serogroup B strain 8047 in which the NspA gene has been deleted (8047 Δ NspA) causes bacteremia and death in infant rats challenged i.p.

Strain	Bacterial dose (CFU)/rat	Results of blood culture 18 h after challenge	
		No. positive/total	CFU/ml (geometric mean, 10^3)
8047 (wild type)	7.8×10^3	3/3	123
8047 Δ NspA	8.3×10^3	3/3	109
8047 Δ NspA	8.3×10^6	3/3 ^a	ND ^b

^a Three of three were dead at 18 h.

^b ND, not done.

there was no evidence of protection 18 h after challenge with strain M986, despite premixing the bacteria with the MAb immediately before the challenge. Although not shown in Table 3, in experiment 3, blood cultures were also obtained 6 h after challenge. At this time point, all animals had bacteremia but some protection was present in animals treated with the 50- μ g dose of AL12 (geometric mean CFU per ml of blood of 1×10^3 versus 34.7×10^3 in the control group which was given the irrelevant MAb; $P = 0.0008$).

Virulence of strain 8047 in which the *nspA* gene has been disrupted. Table 4 summarizes the results of challenging infant rats with strain 8047 Δ NspA. All three animals given a challenge dose of approximately 8×10^3 CFU of strain 8047 Δ NspA had bacteremia present 18 h later. The geometric mean CFU/ml for these pups was similar to that observed in control rats challenged with the 8047 wild-type strain. All three animals challenged with a 10^6 -CFU dose of strain 8047 Δ NspA were dead at 18 h postchallenge.

TABLE 3. Ability of anti-NspA MAb AL12 to passively protect infant rats challenged i.p. with different meningococcal B strains

Experiment no.	Strain (challenge dose per rat in CFU)	MAb pretreatment ^a	Dose (μ g) per rat	Results of blood culture at 18 h	
				No. positive/total	CFU/ml (geometric mean, 10^3) ^b
1	8047 (2.3×10^3)	Anticapsular	10	2/7	0.05
		AL12 Anti-NspA	50	0/7	<0.01
		AL12 Anti-NspA	5	4/7	1.4
		Irrelevant	50	7/7	115
		PBS-albumin	None	7/7	79
2	BZ232 (15.0×10^3)	Anticapsular	50	0/5	<0.01
		Anticapsular	10	0/5	<0.01
		AL12 Anti-NspA	50	4/5	0.2
		AL12 Anti-NspA	10	4/5	0.5
		Irrelevant	50	5/5	11.5
		PBS-albumin	None	5/5	3.9
3	M986 (2×10^3)	Anticapsular	2	0/6	<0.01
		AL12 Anti-NspA	50	5/5	>500
		AL12 Anti-NspA	2	6/6	>500
		Irrelevant	2	5/5	>500

^a In experiments 1 and 2, animals were pretreated with the MAb i.p. at time zero. Two hours later, the animals were challenged i.p. with bacteria. In experiment 3, to increase the sensitivity of the assay, the bacteria and MAb were mixed together immediately before the i.p. challenge. In this experiment, blood was also obtained for culture at 6 h. Results for this time are provided in the text.

^b For calculation of geometric mean CFU per milliliter, animals with sterile cultures were assigned a value of 10 CFU/ml. The geometric mean CFU per milliliter of animals receiving AL12 were compared to that of the combined group of negative-control animals given irrelevant MAb or PBS-BSA: experiment 1, 50 μ g of AL12 versus controls, $P < 0.001$; 5 μ g of AL12 versus controls, $P < 0.005$; experiment 2, 50 μ g of AL12 versus controls, $P < 0.01$; 10 μ g of AL12 versus controls, $P < 0.01$.

DISCUSSION

The results presented here extend our previous observations made with polyclonal antisera (16). Using a panel of anti-NspA MAbs, we have shown that large differences exist among *N. meningitidis* serogroup B strains in the ability of anti-NspA antibody to bind to the surface of live encapsulated bacteria, to elicit complement-mediated bacteriolysis, and to confer passive protection in vivo. With the exception of two strains, the results were highly reproducible in replicate experiments. The two exceptional strains, BZ232 and MCS8, showed variations in anti-NspA-surface binding among different subcultures (Fig. 4). Another important new finding is the ability of an NspA-knockout strain to cause bacteremia and death of infant rats after i.p. challenge (Table 3), a result indicating that NspA expression is not essential for causing bacteremia.

The underlying mechanism(s) responsible for the observed strain differences in anti-NspA surface binding remains unknown. Although NspA is highly conserved among *N. meningitidis* serogroup B strains (4, 13, 16), changes in one or more amino acids in the protein, particularly in surface-accessible loops, could result in decreased anti-NspA binding. However, with one notable exception (4), all strains in which the *nspA* gene has been sequenced show high conservation of gene segments encoding putative surface-exposed loops (100% identity for *N. meningitidis* serogroup B strains) (4, 16). Also, strains NGP165 and CU385 have nearly identical protein sequences (one amino acid difference, Gly versus Ala at position 150, which is not located in a putative loop segment), yet NGP165 is negative for surface binding with AL12 while CU385 is strongly positive (16). Finally, based on colony Western blot analysis, the NspA epitope defined by MAb AL12 was present in all 10 strains examined, despite negative or variable surface binding in four of the strains (Table 2). Therefore, amino acid sequence variation is unlikely to result in the differences in functional activity of the anti-NspA MAbs described in this study.

Alternative mechanisms that might explain decreased surface accessibility of NspA on live bacterial cells and/or decreased susceptibility to complement-mediated anti-NspA bacteriolysis include strain differences in the amount of NspA produced and steric interference by other variable bacterial surface structures or a combination of the two. In our previous study with polyclonal anti-NspA antisera, we found a statistically significant trend for poorer antibody surface binding among the *N. meningitidis* serogroup B strains that were the highest producers of capsular polysaccharide (16). However, some strains were inconsistent with this trend. For example, strain 8047 is a high producer of capsular polysaccharide but is positive for anti-NspA surface binding and susceptible to bactericidal activity, while strain NGP165, which is a low capsular polysaccharide producer, is negative for anti-NspA surface binding and resistant to bactericidal activity. Some exceptions can be explained by quantitative differences in NspA expression (i.e., high for 8047 and low for NGP165, as determined by SDS-PAGE of lauroyl sarcosinate-extracted outer membrane preparations [16]). For other strains such as CU385 that were positive for surface binding but negative for bactericidal activity and other strains with variable anti-NspA reactivity, such as MCS8 and BZ232, there were no apparent differences in NspA

expression. Therefore, there are likely to be other mechanisms for interfering with binding and bactericidal activity.

The data indicating variation in anti-NspA-surface binding in respective subcultures of two strains are of particular interest (Fig. 4), given that NspA expression was uniform in all CFU tested by Western blotting. Furthermore, colonies derived and expanded from each of the three subcultures could test positive or negative for NspA surface binding by the flow assay. In contrast, there was no variability of anti-PorA MAb binding to the bacterial cells from the same strains in control experiments (Fig. 4). These results imply that some other variable surface structure can inhibit antibody binding to NspA but not to PorA. Future studies will need to examine the possible effect of lipooligosaccharide phenotypes, expression of other outer membrane proteins, or sialylation of surface molecules on antibody binding to NspA epitopes.

Whatever the underlying mechanism(s), the observed strain and intrastrain variability in NspA surface accessibility appears to have important consequences for the ability of anti-NspA antibodies to elicit complement-mediated bacteriolysis. None of the strains that were negative or variable for surface binding in the flow cytometry assay were susceptible to anti-NspA complement-mediated bacteriolysis (Table 2). There were also two strains (NG3/88 and CU385), each of which was positive for surface binding with the four MAbs but resistant to complement-mediated bacteriolysis, even when tested at >10-fold-higher antibody concentrations than those used in the surface binding assay (Table 2). In the presence of complement, both strains were readily killed by the positive-control IgG anticapsular antibody. The reasons for poor bactericidal activity of the anti-NspA MAbs that bind well to these bacteria remain unknown.

The passive protection data obtained with MAb AL12 in infant rats challenged by different *N. meningitidis* serogroup B strains were consistent with the in vitro bactericidal results (compare Tables 2 and 3). Thus, MAb AL12 was highly protective against challenge by *N. meningitidis* serogroup B strain 8047 (surface binding positive and susceptible to bacteriolysis), was partially protective against strain BZ232 (surface binding variable and resistant to bacteriolysis), and failed to protect against strain M986 (negative for surface binding and resistant to bacteriolysis).

The bactericidal and passive protection results described above differ from those described by Cadieux et al., who reported that an anti-NspA MAb, Me-7, was broadly bactericidal against a panel of 14 serogroup B, C, and A strains (all but 1 serogroup A strain were killed) (4). Since we were unable to obtain MAb Me-7 from Cadieux et al. for our studies, we cannot exclude the possibility that this MAb is more broadly reactive and bactericidal than the MAbs evaluated in our panel. However, an alternative explanation for these conflicting results may lie in the differences in the methods used to assess bactericidal and passive protective activities. For example, Cadieux et al. defined protection in their mouse model as a significant decrease in bacteremia 5 h after challenge in the treated animal. Typically, the Me-7-treated mice that were considered protected had 10^3 to 10^4 bacteria per ml of blood (<25% of control animals). In contrast, the primary endpoint of protection in our experiments was bacteremia measured at 18 h. In a passive protection experiment, antibody amount is

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limited. Therefore, it is possible to observe protection at 6 h but not at 18 h after challenge, as observed with strain M986 (see Table 3 and Results).

In conclusion, considerable evidence indicates that NspA is highly conserved in pathogenic *Neisseria meningitidis* strains and that NspA is expressed in all *N. meningitidis* serogroup B strains tested to date (4, 13, 16). The present data with a panel of anti-NspA MAbs confirm earlier observations with polyclonal antisera that despite *nspA* gene conservation and expression, many *N. meningitidis* serogroup B strains show poor antibody binding to the surfaces of live bacteria and resist anti-NspA complement-mediated bacteriolysis. Further studies are needed to define the underlying mechanisms modulating surface accessibility of NspA. The ability of the NspA gene knockout strain to cause bacteremia and lethality in infant rats (Table 4) provides evidence that this molecule is not essential for serum resistance or for causing bacteremia once the organism has entered the blood stream. Taken together, the data underscore the likelihood that an NspA-based vaccine will need to be supplemented by additional antigens to elicit broad-based protection.

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Binding diversity of monoclonal antibodies to $\alpha(2 \rightarrow 8)$ polysialic acid conjugated to outer membrane vesicle via adipic acid dihydrazide

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Abstract

Murine monoclonal antibodies (mAbs) were generated using group B *Neisseria meningitidis* and *Escherichia coli* K1 polysaccharides (PSs) conjugated to outer membrane vesicle (OMV) via adipic acid dihydrazide, and were used to identify the immunodeterminants expressed on these capsular PSs. Ten mAbs representative of IgM and all subclasses of IgG were obtained which recognized diverse immunodeterminants on $\alpha(2 \rightarrow 8)$ polysialic acid (PSA). The specificity of mAbs to different antigenic determinants was assessed by their differential binding to PSA attached to a solid phase by different methods and confirmed by absorption studies. Two mAbs from the *E. coli* K1 fusion were directed to the *O*-acetyl epitope and the rest reacted with both the PSs only when attached to a solid phase by certain means. The methods by which PSA was coated on the solid phase had an impact on the epitope expression and binding pattern. At the concentrations used, the *O*-acetyl-specific mAbs, IgG1 and IgG3 mAbs were not bactericidal against group B *N. meningitidis*, whereas other mAbs were. The conjugates B and K1 PSs present to the murine immune system different antigenic determinants, some of which elicit bactericidal antibodies.

Keywords: Conjugate vaccine; Monoclonal antibody; Polysialic acid; Epitope

1. Introduction

$\alpha(2 \rightarrow 8)$ polysialic acid (PSA) forms the capsule of group B *Neisseria meningitidis*, *Escherichia coli* K1, *Moraxella nonliquefaciens* and serotype 2 of *Pasteurella haemolytica* [1–4]. PSA also forms one

half of the alternating repeat unit of the capsule of *E. coli* K92 [5]. Both group B *N. meningitidis* and *E. coli* K1 continue to cause fatal systemic infections in humans including meningitis. PSA facilitates bacterial survival in the host by its antiphagocytic and anticomplementary actions. PSA has been extensively studied because of its conformational complexity, poor immunogenicity and its presence on developing mammalian neural and renal tissues [6,7].

The increased concern over group B *N. meningitidis* and *E. coli* K1 infections has heightened inter-

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est in the generation of anti-capsular monoclonal antibodies (mAbs) of functional significance. MAbs capable of probing various epitopes of the PSA molecule are valuable for defining determinants of biologic or immunopathologic significance. Because of the poor immunogenicity of the purified PSA, mAbs reactive with PSA have been induced previously only by infecting NZB or BALB/c mice with live group B *N. meningitidis* or *E. coli* K1, or by immunization with formalin-killed cells [8-12]. Most of these mAbs were IgM and few were IgG [8,9].

The importance of PSA in pathogenesis and protection of group B *N. meningitidis* and *E. coli* K1 infections has led scientists to render PSA immunogenic. The initial work on conjugating unmodified PSA to tetanus toxoid failed in eliciting PSA-specific antibodies [13]. However, later attempts led to the development of immunogenic conjugates with or without chemical modification of PSA [14-16]. The properties of antibodies, both polyclonal and monoclonal, induced by the chemically modified (*N*-propionylated) group B meningococcal polysaccharide (B PS)-tetanus toxoid (TT) conjugate have been evaluated; these antibodies were shown to be specific for a bactericidal epitope on the surface of group B *N. meningitidis* [17-19]. However, the properties and the biologic activities of antibodies to group B or *E. coli* K1 PS conjugated to a protein carrier via adipic acid dihydrazide (ADH) have not been reported previously. We describe here the binding specificity, isotype composition, and bactericidal properties of two panels of mAbs generated by immunizing BALB/c mice with a group B meningococcal PS-outer membrane vesicle (B-OMV) or an *E. coli* K1-OMV (K1-OMV) conjugate vaccine.

2. Materials and methods

2.1. Strains and materials

Group B *N. meningitidis*, strain M986 (B:2a:P1.2) used for PS preparation and bactericidal assays was obtained from the culture collection of the Laboratory of Bacterial Polysaccharides, Center for Biologics Evaluation and Research (CBER). *O*-Acetyl positive *E. coli* K1 (*O*-Ac⁺), strain 156, and *E. coli* K92, strain Bos were obtained from Dr. Willie Vanu,

CBER. Polynucleotide A, sodium borohydride, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, denatured DNA and ADH were from Sigma Chemical Co., St. Louis, MO. The Ribi adjuvant (monophosphoryl lipid A and trehalose dicorynomycolate) was purchased from Ribi Immunochem Research, Inc., Hamilton, MT.

2.2. Polysaccharides and proteins

The group B meningococcal, and *E. coli* K1 and K92 PSs were extracted and purified according to the standard procedure [3,20]. The *Haemophilus influenzae* b (Hib) and *O*-Ac⁺ group C meningococcal PSs were obtained from Merck Research Laboratories, West Point, PA.

N-deacetylated and *N*-propionylated group B meningococcal PSs were prepared as described by Jennings et al. [14]. Removal of *N*-acetyl groups and replacement with *N*-propionyl groups was confirmed by ¹³C nuclear magnetic resonance spectroscopy (kindly performed by Dr. William Egan, CBER).

Group B meningococcal OMV from a non-capsular variant (strain M986-NCV1) was prepared and purified as described [21]. TT was obtained from Connaught Laboratories Inc., Swiftwater, PA. Human serum albumin (HSA) (25%) was obtained from the Michigan Department of Public Health, Lansing, MI.

2.3. Conjugates

Group B meningococcal and *E. coli* K1 PSs were conjugated to OMV (B-OMV and K1-OMV) after introducing the spacer, ADH, by using a modification [15] of the procedure described earlier [16]. Derivatization of the PS and conjugation to TT were carried out at pH 6.5 in 0.2 M NaCl, while conjugation with OMV was performed in sterile deionized water adjusted to pH 6.8, which helped to keep OMV soluble [15].

2.4. Immunization of mice

A group of five BALB/c mice were immunized twice i.p. with 5-10 µg per mouse of the desired conjugate vaccine. The K1-OMV conjugate was injected first with the Freund's complete and then with the incomplete adjuvant, four weeks apart. The B-

OMV conjugate was injected to mice twice with the Ribi adjuvant three weeks apart. All mice showed high levels of serum IgG and IgM antibodies to group B meningococcal and/or *E. coli* K1 PSs by an ELISA described below [22]. After a resting period of eight and four weeks respectively, the mice were boosted intravenously with the same conjugate without the adjuvant. Three days later, cell fusions were performed according to the method described [23] using mouse SP2/O-Ag 14 myeloma cells and the standard polyethylene glycol technique.

2.5. ELISA

Initial screening of supernatants with group B meningococcal and/or *E. coli* K1 PS was performed using a previously described ELISA which used the purified PSA complexed with methylated HSA (mHSA) as the coating antigen [15,22]. Some supernatants were also screened using the ADH-containing B-HSA conjugate as the coating antigen; an ADH-specific mAb was obtained during this secondary screening. Linear binding curves were obtained for each mAb concentrate using doubling dilutions starting from 1:2, and the OD₄₉₃ was measured using a microplate autoreader (EL309, BioTek Instruments). Concentrated super media [23] was used to assess the background ELISA reading, which was subtracted from the OD₄₉₃ of each mAb.

2.6. Clone selection

The supernatants from original colonies that showed moderate to high antibody readings with the group B meningococcal and/or *E. coli* K1 PS by ELISA were identified and those cell colonies were cloned by limiting dilutions. Supernatants of all clones were rescreened by ELISA and those showing reactivity with either of the PSs were produced in large quantities and concentrated to 15–20 times their original volume using minicon-B15 concentrators (Amicon, Beverly, MA).

2.7. Isotype determination and immunoglobulin quantitation

Isotype composition of mAbs was determined using the Immuno Select ELISA kit (Gibco BRL, Bethesda, MD).

The quantity of immunoglobulin in each concentrated hybridoma supernatant was determined using a radial immunodiffusion kit (Southern Biotechnology Associates, Birmingham, AL). Reference standards provided by the manufacturer were used.

2.8. Agglutination, precipitation and immunodot

The ability of mAbs to agglutinate the cell suspensions of group B *N. meningitidis* and *E. coli* K1 was tested on microslides. The precipitability of mAbs with native and chemically modified PSA and with non-PSA antigens was examined by Ouchterlony immunodiffusion and/or immunodot; the antigens were used at a concentration of 1 mg ml⁻¹.

The specificity of mAbs to the spacer, ADH, and to the denatured DNA was examined by immunodot or by ELISA. K1-TT, B-HSA, C-TT and 92-TT were the ADH-containing sialic acid conjugate antigens that were used in the assay. An ADH-containing non-sialic acid conjugate antigen composed of the capsular PS of *Vibrio vulnificus* and TT (VV-TT) was also included [25].

2.9. MAb binding assays

Direct binding assays were performed in Immulon 1 microwell plates (Dynatech Laboratories, Chantilly, VA) using different antigenic forms of PSA. Specific binding of mAbs was determined by ELISA using PSA and non-PSA antigens (Table 1). Non-PSA antigens included: the capsular PSs of group A *N. meningitidis*, Hib and *E. coli* K13, all attached to microwell plates in a two-step coating procedure via poly-L-lysine (PLL) as described [24]. The PSA antigens included group B meningococcal and *E. coli* K1 PSs attached to solid phase in a single step coating procedure by complexing with mHSA, or via PLL pre-coated on microwell plates, or after covalent conjugation with HSA or with TT via ADH. *E. coli* K92 and group C meningococcal PSs were attached after conjugation to TT via ADH. Modified PSA, i.e. *N*-deacetylated and *N*-propionylated group B meningococcal PSs, and polynucleotide A were attached to ELISA plates via pre-coated PLL.

Display of different epitopes by PSA when bound to the solid phase by different methods was confirmed in a two-set ELISA by immobilizing various

Table 1
Structure of sialic acid and non-sialic acid capsular PSs used in binding assays

Bacteria	Repeating unit
Group B <i>Neisseria meningitidis</i>	8)- α -N-acetyl neuraminic acid-(2 $\rightarrow$
Group C <i>Neisseria meningitidis</i>	9)- α -N-acetyl neuraminic acid-(2 $\rightarrow$
<i>Escherichia coli</i> K1	8)- α -N-acetyl neuraminic acid-(2 $\rightarrow$, OAc ⁺
<i>Escherichia coli</i> K92	8)- α -N-acetyl neuraminic acid-(2 $\rightarrow$ 9)- α -N-acetyl neuraminic acid-(2 $\rightarrow$
Group A <i>Neisseria meningitidis</i>	6)- α -N-acetyl mannosamine phosphate-(1 $\rightarrow$
<i>Haemophilus influenzae</i> type b	3)- β -D-ribose-(1 $\rightarrow$ 1)-D-ribitol-5-(phosphate $\rightarrow$
<i>Escherichia coli</i> K13	3)- β -ribose-(1 $\rightarrow$ 7)- β -KDO-(2 $\rightarrow$, OAc ⁺

antigenic forms of PSA on solid phase as reported previously [22]. An optimal dilution of the mAb that fell in the upper part of the linear dilution curve was pre-incubated with various concentrations of native group B meningococcal and/or *E. coli* K1 PS attached to the first microtiter plate by different methods described above. The pre-incubated mAb dilution was then transferred to a second microwell plate coated with group B meningococcal or *E. coli* K1 PS complexed with mHSA, and ELISA continued as described above. The OD₄₉₅ of each pre-absorbed mAb obtained from the second ELISA plate was compared with the OD₄₉₅ of unabsorbed sample of the same mAb; the latter was considered as 100% binding and the percent reduction in binding calculated.

2.10. Temperature sensitivity

The effect of temperature on mAb binding was examined by performing ELISA at room temperature and at 37°C; the latter was performed in a warm room maintained at 37 $\pm$ 2°C using pre-warmed reagent solutions. All mAbs except those specific for the O-Ac epitope were tested at a single dilution that was chosen to maintain antigen excess.

2.11. Bactericidal assay

A microbactericidal method [21] was followed using rabbit complement (Pel-Freez, Brown Dear, WI) and group B *N. meningitidis*, strain M986.

3. Results

Based upon the poor experience in the past of eliciting IgG antibodies to the native PSA, we inves-

tigated Freund's or Ribi adjuvants for their ability to induce mAbs of diverse isotypes to various PSA epitopes. Fusion A derived from mice immunized with the K1-OMV conjugate in Freund's adjuvant yielded IgM mAbs and mAbs of all IgG subclasses. Fusion B derived from mice immunized with the B-OMV conjugate in the Ribi adjuvant resulted in IgM, IgG1, IgG3 and IgG2a mAbs. The mAbs were identified by their isotype as shown in Table 2; their numerical designation contains the letter A or B to indicate the specific fusion from which they were generated.

3.1. Binding specificity

Table 2 shows the binding pattern of each mAb to a panel of different antigenic presentations of group B meningococcal and *E. coli* K1 PSs. The K1 PS contains O-acetyl groups, while the B PS does not. IgG1-A₃ and IgG3-A₁, generated by the K1-OMV conjugate appeared to be O-Ac-specific; the rest were reactive with *E. coli* K1 and group B meningococcal PSs attached to solid phase in one form or the other. On the other hand, all mAbs generated by the B-OMV conjugate had specificity for both group B meningococcal and *E. coli* K1 PSs.

Except for IgG2b-A₄, there was no binding of mAbs to heterologous, non-sialic acid-containing PSs of group A *N. meningitidis*, Hib and *E. coli* K13, or to N-deacetylated or N-propionylated B PS as examined by ELISA (using PLL system), immunodot and Ouchterlony gel diffusion. This suggested that most of the mAbs were specific for epitopes on the native B and K1 PSs. IgG2a-B₁₀ and the IgM mAbs showed weak binding to PNA attached to plates pre-coated with PLL.

In addition to reacting with the conjugate antigens, IgG1-B₉ showed binding to non-conjugated

Table 2
Binding specificity^a of mAbs derived from B-OMV and K1-OMV conjugate vaccines

Isotype	ELISA coating antigen ^b								
	K1 + mHSA	B + mHSA	K1-TT	B-HSA	K92-TT	C-TT	B + PLL	K1 + PLL	PNA ^d
IgM-A ₁	+++ ^b	– ^c	+++	+++	–	–	++	++	+
IgM-A ₂	+++	±	+++	++	–	–	–	+++	±
IgG1-A ₃	+++	–	++	–	–	–	–	+	–
IgG2a-A ₄	+++	–	++++	+	–	–	–	ND	–
IgG2b-A ₅	+++	++	+	++	–	–	–	–	–
IgG2b-A ₆	–	–	++++	++++	++++	++++	–	–	ND
IgG3-A ₇	+	–	++	–	–	–	–	+	–
IgM-B ₈	+++	+++	++++	+++	–	–	+++	–	±
IgG1-B ₉	+++	+++	+++	+	+++	+	–	–	–
IgG2a-B ₁₀	+++	++	++++	++	–	–	–	–	±
IgG3-B ₁₁	++	++	++	–	–	–	–	–	–

^a The letter designation at the end of each mAb indicates the fusion from which each mAb was derived: A: K1-OMV vaccine and B: B-OMV vaccine.

^b None of the mAbs except IgG2b-A₆ reacted with non-sialic acid-containing polymers or with denatured DNA.

^c OD₄₉₅: 0.050 to 0.099, ±: 0.100 to 0.299, +: 0.300 to 0.499, ++: 0.500 to 2.000, +++: > 2.000, +++++.

^d Negative.

^e Polymaclosetide A.

^f IgG2b-A₆ reacted with an ADH-containing non-sialic acid-containing conjugate (VV-TT), whereas IgG1-B₉ did not.

group B meningococcal and *E. coli* K1 antigens mixed with mHSA, but not with the same antigens attached to plates pre-coated with PLL. IgM-A₂, IgG2a-A₄, IgG2a-B₁₀ and IgG1-B₉ did not bind to B-HSA conjugate antigen as strongly as they did to *E. coli* K1-TT conjugate or K1 + mHSA antigens. While all mAbs of fusion A except IgG2a-A₄, IgG2b-A₅ and IgG2b-A₆ reacted with group B meningococcal and/or *E. coli* K1 PS attached via PLL, only one mAb from fusion B, i.e. IgM-B₈, showed such binding.

Representative binding curves of mAbs obtained when K1 + mHSA complex was used as the coating antigen are shown in Fig. 1. The difference in binding of IgM-A₂ compared to that of IgG2a-A₄ despite the higher concentration of the latter, was suggestive possibly of the higher avidity of the former mAb. The binding pattern of four representative mAbs to different antigenic forms of B and K1 PSs was noted (Table 3). At the concentrations used, IgG2a-B₁₀ and IgM-B₈ required only 5–8 ng ml^{–1} of K1 antigen conjugated to TT to produce an OD₄₉₅ of 1.0, compared to 228 and 24 times more of K1 antigen complexed with mHSA to produce a binding of similar degree. IgM-A₂ required about four-times more K1-TT conjugate than K1 + mHSA antigen to show similar binding.

Fig. 2A shows the binding curves of a representative IgM-B₈ mAb obtained with different antigenic presentations of B and K1 PSs when examined as a function of antigen concentration. Fig. 2B and C show reduction in binding of IgM-B₈ on a B + mHSA coated plate after pre-incubation with different antigenic forms of B and K1 PSs. The percent reduction in binding produced by B and K1 PSs

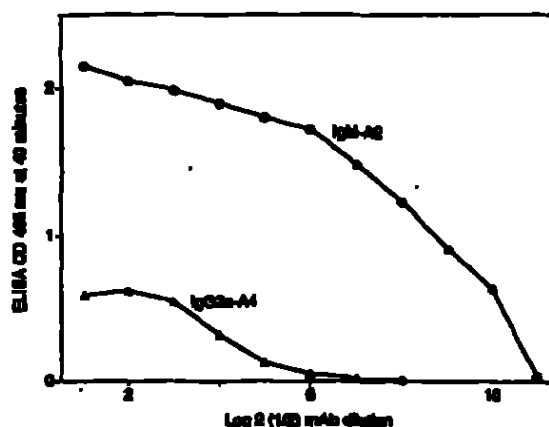


Fig. 1. Direct binding of mAb IgM-A₂ and IgG2a-A₄ to K1PS + mHSA-coated ELISA plate. Antibody concentrations of IgM-A₂ and IgG2a-A₄ were 139 µg ml^{–1} and 4900 µg ml^{–1}, respectively.

82.1

Table 3
Binding diversity of representative mAbs to PSA^a

mAb	μg of antigen ^b needed to produce an OD ₄₉₂ of 1.0						
	K1 ^a	K1 + mHSA	K1-TT ^b	B ^a	B + mHSA	B-HSA ^b	Other antigens ^c
IgM-A ₂	1.26	0.82	3.22	> 30.00	24.2	3.4	> 30.0
IgM-B ₂	> 30.00	1.90	0.08	3.10	2.63	1.5	> 30.0
IgG2a-B ₁₀	> 30.00	11.40	0.05	> 30.00	4.80	12.2	> 30.0
IgG2b-A ₃	> 30.00	13.10	28.20	> 30.00	29.80	27.4	> 30.0

^a MAbs were tested at an optimal dilution that fell in the upper part of the linear dilution curve, and therefore the testing concentration of different mAbs is not same.

^b Values represent the mean of duplicate runs.

^c Attached via PLL.

^d ADEH-containing conjugate antigen.

^e *N*-propionylated and *N*-deacetylated B PSa, colominic acid and polynucleotide A attached via PLL; ADEH-containing C-TT and K92-TT conjugate antigen.

bound to the plate by different means varied widely. For instance, IgM-B₂ required more B PS coated with PLL and mHSA (23.9 and 3.4 $\mu\text{g ml}^{-1}$ respectively) to show a 50% reduction in binding (Fig. 2B), whereas only 0.46 $\mu\text{g ml}^{-1}$ of the K1 PS in a conjugate was sufficient to produce similar percent

reduction (Fig. 2C). This observation may be due to higher epitope density on the PSa bound to the solid phase in a conjugate form. The results of binding studies led to categorize mAbs into several groups of microspecificity on the basis of their variable binding patterns (Table 4).

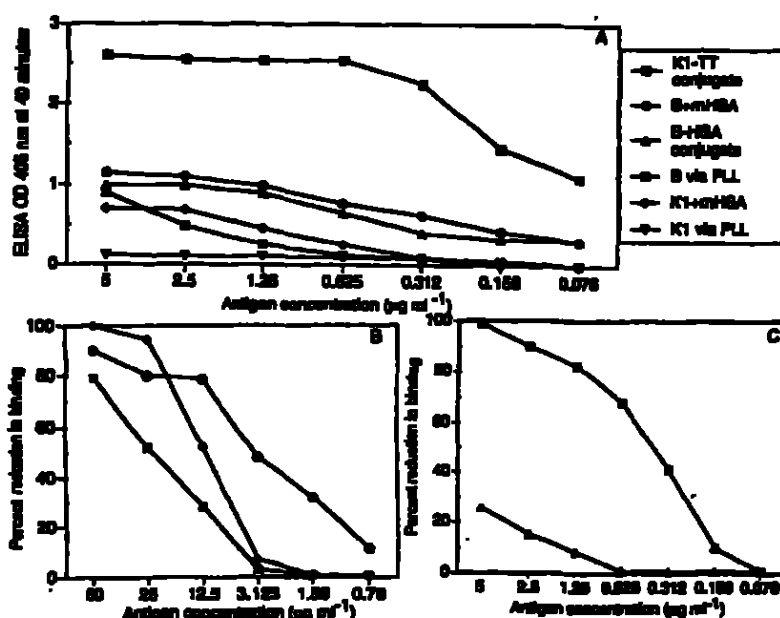


Fig. 2. Patterns of antibody binding of a representative mAb, IgM-B₂, as measured by ELISA before and after pre-incubation with various antigenic presentations of PSA. PSa were used in concentrations that ranged from 0.78 to 30.0 $\mu\text{g ml}^{-1}$ and conjugate antigens in concentrations of 0.78 to 30.0 $\mu\text{g ml}^{-1}$. IgM-B₂ at a concentration of 1.2 $\mu\text{g ml}^{-1}$ was first allowed to bind with different PSA presentations coated to a microwell plate as indicated (A). MAb dilution from each well was then transferred to a second set of microwell plates coated with 5 $\mu\text{g ml}^{-1}$ of B + mHSA and percent reduction in binding plotted (B and C) as described in the text.

Table 4
Bactericidal activity^a and microheterogeneous specificity to mAbs to PSA

Isotype	Binding specificity	MAb concentration ($\mu\text{g ml}^{-1}$) at 70% killing
IgM-A ₁	K1 + mHSA, K1-TT, B-HSA > K1 and B via PLL	ND
IgM-A ₂	K1 + mHSA, K1-TT, K1 via PLL > B-HSA > B + mHSA	34.8
IgG1-A ₃	K1 + mHSA, K1-TT > K1 via PLL	> 8.3
IgG2a-A ₄	K1-TT > K1 + mHSA > B-HSA	1130.0
IgG2b-A ₅	K1 + mHSA > B + mHSA, B-HSA > K1-TT	282.5
IgG3-A ₇	K1-TT > K1 + mHSA, K1 via PLL	> 1500.0
IgM-B ₈	K1-TT > B + mHSA, B-HSA, B via PLL > K1 + mHSA	18.7
IgG1-B ₉	K1 + mHSA, B + mHSA, K1-TT, K92-TT > B-HSA > C-TT	> 254.0
IgG2a-B ₁₀	K1-TT > K1 + mHSA > B-HSA, B + mHSA	1330.0
IgG3-B ₁₁	K1 + mHSA, B + mHSA, K1-TT	> 1500.0

^a Rabbit complement and group B *N. meningitidis* strain M986 were used.
ND, not determined.

3.2. Temperature sensitivity

Since group B PS antibodies have been previously shown to react better with the B PS at lower temperatures [12], we investigated the effect of temperature on binding of mAbs. While IgM mAbs showed a 24–49% decrease in binding at 37°C relative to room temperature, the decrease in binding of IgG mAbs was 5% or less.

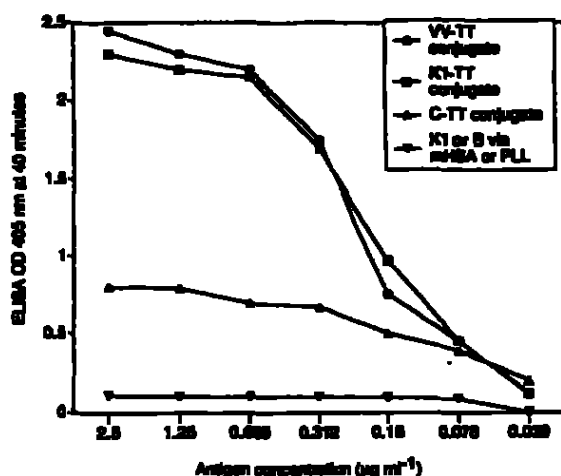


Fig. 3. Binding pattern of a representative ADH-specific mAb (IgG2b-A₅) with increasing concentrations of several ADH-containing antigens bound to the microwell plate.

3.3. Agglutination, precipitation and immunodot

IgM-B₈ agglutinated saline cell suspensions of both group B *N. meningitidis* and *E. coli* K1. Two IgG mAbs, IgG3-A₇ and IgG2a-B₁₀, agglutinated cells of *E. coli* K1 only. A similar positive precipitation reaction was exhibited by these mAbs with the B and K1 PSs (not shown). None of the precipitating mAbs reacted with non-PSA antigens or with modified B PSs, polynucleotide A and denatured DNA.

The ADH-specificity of an IgG2b mAb, i.e. IgG2b-A₅, was confirmed by its binding both to PSA and non-PSA conjugates containing ADH (Fig. 3). This mAb reacted with K1-TT, C-TT, K92-TT and B-HSA conjugates and also with a non-PSA conjugate, VV-TT, but failed to bind to K1 + mHSA, B + mHSA, K1 + PLL or B + PLL antigens by ELISA.

3.4. Bactericidal activity

The bactericidal activities of mAbs against group B *N. meningitidis* as tested in presence of rabbit complement are shown in Table 4 in relation to their binding specificities. IgM mAbs were bactericidal at lower concentrations than IgG mAbs. The bactericidal titers of IgG2a and IgG2b mAbs varied and IgG3 mAbs were not bactericidal at a concentration of 1500 $\mu\text{g ml}^{-1}$. IgG1 mAbs and those with specificity for O-Ac epitope, or for ADH were also not

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bactericidal at the concentrations used. No definite correlation could be found between binding pattern and bactericidal activity of mAbs.

4. Discussion

We generated murine mAbs to B or K1 PSs after rendering them immunogenic by conjugating to group B meningococcal OMV via ADH, and evaluated their determinant specificity and in vitro functional activity. B-OMV and K1-OMV conjugates elicited ten mAbs representative of IgM and all subclasses of IgG. We determined the differential binding specificity of B and K1 specific mAbs by attaching the PSs to ELISA plate using mHSA, pre-coated PLL, or after conjugation with HSA or TT.

MAbs exhibited microheterogeneity in epitope binding. The strongest antibody binding was observed with the B or K1 PS covalently coupled to protein carriers or non-covalently complexed with mHSA. IgG2a-A₄ showed binding to B-HSA conjugate, but not to unconjugated B PS attached to ELISA plate using PLL or mHSA. The K1 PS attached via PLL showed binding with IgM-A₁, IgM-A₂, IgG1-A₃ and IgG3-A₇ derived from K1-OMV conjugate. The B PS attached by this means reacted with only one IgM mAb from either fusion (IgM-A₁ and IgM-B₁). It appears that several epitopes reside on the B PS, some of which may attain stable antigenic configuration and become accessible for mAb binding when conjugated to a protein carrier. The space-filling model of PSA published by Michon et al. [29] has revealed the disposition of carboxyl and acetamido groups on opposite sides of the molecule. Structural studies of PSA have indicated the presence of non-linear helical, immunologically functional conformational epitopes on PSA [26–31]. It is possible that the reactive epitope is stabilized by the process of conjugation and may involve the reported complex secondary structures of the B PS [30–32]. Further studies are needed to understand the possible relationship between the variability in binding of mAbs and the maintenance of the critical conformational structure of the B or K1 PS, or the internal flexibility of the polymer when presented in certain antigenic forms on the solid phase.

Generation of mAbs of O-Ac-specificity by fusion A (derived from the K1 conjugate) suggested that the O-Ac epitope is recognized by the murine immune system as a dominant epitope in an otherwise poorly immunogenic molecule. These mAbs may be useful in definitive typing of clinical and veterinary isolates of *E. coli* K1. It is interesting that IgG1-A₃, IgG2a-A₄, IgG3-A₇ mAbs reacted with one or the other antigenic presentation of unconjugated K1 PS indicating the possible conformational stability conferred to the PSA backbone by O-Ac groups. MAbs derived from fusion B reacted with both K1 and B PSs suggesting that they both share common epitopes.

The ability of mAbs to bind to B or K1 PS was assessed both as a function of antigen and antibody concentration. The variation in intensity of direct binding of different mAbs to different antigenic forms of PSA may be related to the epitope density presented by the PSA based on the configuration in which it is attached to the solid phase. A comparison of the binding profiles as a function of mAb concentration indicated that the relative binding capacity of IgG mAbs was lower than that of IgM mAbs. The intensity of binding increased when B or K1 PS covalently linked to a protein, or complexed with mHSA, was used as the coating antigen. The results indicate that establishment of appropriate coating methods that retain the antigenic integrity of PSA may be essential. With regard to the microspecificity, the results obtained with the mAbs are consistent with those reported with infection-induced B PS-specific IgM mAb [10] and PS- or conjugate-induced C PS-specific mAbs [33]. No definite correlation could be established between epitope specificity and bactericidal activity of mAbs. IgG mAbs were bactericidal at much higher concentrations than IgM mAbs and this may be related to their poor avidity, similar to that observed with human antibodies elicited by Hib PS conjugated to meningococcal OMP complex [34].

IgG1-B₁ and IgG2b-A₆ reacted with ADH-containing PSA conjugate antigens, i.e. K1-TT, B-HSA, C-TT and K92-TT. The PSA-specificity of the former and the ADH-specificity of the latter was demonstrated by reacting them also with an unrelated ADH-containing non-PSA conjugate, VV-TT [25]. Only IgG2b-A₆ reacted with the VV-TT conju-

gate antigen. The observation that B- and K1-OMV conjugates induce two populations of mAbs, one with specificity for the PS and the other with specificity for an epitope associated with ADH, is consistent with the observation made with polyclonal antibodies induced by B and K92 PS conjugates [15,35]. The ADH-specific IgG2b-A₆ mAb was non-bactericidal against group B *N. meningitidis*.

Some infection-induced murine mAbs or gammopathy-induced human mAbs with specificity for PSA have shown binding to different polynucleotides [36,37]. In our study, all IgM mAbs and IgG2a-B₁₀ showed a weak, but reproducible binding with polynucleotide A. Only non-O-Ac-specific IgM mAbs showed significant decrease in binding at 37°C, similar to the infection-induced B PS-specific IgM mAbs [12] and conjugate-elicited polyclonal PSA antibodies [16].

In conclusion, B and K1 PSs have distinct antibody recognition sites. B-OMV and K1-OMV conjugates are immunogenic, antigenically complex and present to the murine immune system several immunodeterminants of PSA, some of which appear to elicit bactericidal IgG and IgM antibodies. Conjugate-elicited mAbs are diverse in isotype composition, binding pattern, antigenic specificity and in vitro bactericidal activity. Although we cannot speculate on the exact mechanism by which PSA binds to the plate by different methods used in this study, the antigenic form in which B and K1 PSs are attached to the microtiter plate appears to influence epitope display and/or density. The utility of our mAbs in identification of bacteria that possess PSA as a surface antigen and in capsular typing, in protection against meningococcal and *E. coli* K1 infections in animal models and in immunolocalization of PSA on mammalian tissues are to be explored.

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