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Specificity of Bactericidal Antibody Response to Serogroup B Meningococcal Strains in Brazilian Children after Immunization with an Outer Membrane Vaccine

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Pre- and postvaccination serum samples from 77 children aged 2 to 6 years, who received the Cuban BC vaccine (B:4:P1.15), were analyzed for bactericidal antibodies against a local B:4:P1.15 strain (N44/89). Sera from 16 individuals with bactericidal antibodies against the B:4:P1.15 strain were tested against 23 Brazilian isolates. These include B:4 strains of distinct serosubtypes: P1.15, P1.7,1, P1.3, P1.9, P1.nt, and a B:8,19,23: P1.16 strain. A Cuban B:4:P1.15 strain (Cu385/83) was also included in the study. The specificities of bactericidal antibodies were analyzed by using mutant strains lacking a class 1 protein (PorA protein) or a class 5 protein or both. The results indicated that PorA and class 5 proteins are the main targets recognized by the bactericidal antibodies of vaccinees. Nonetheless, a complex pattern of recognition by bactericidal antibodies was found, and vaccinees were grouped according to antibody specificity. Antibodies from some individuals recognized PorA of serosubtype P1.15. However, antibodies from these individuals could not kill all P1.15 strains tested. Antibodies from a second group recognized both PorA and class 5 proteins, and antibodies from a third group recognized an as yet unidentified target antigen. The results demonstrate the importance of determining the fine epitope specificity of bactericidal antibodies to improve the existing vaccines against B meningococci.

Group B meningococcal disease remains a significant public health problem in Brazil and in many other countries (17, 22). In contrast to polysaccharides A and C, B polysaccharide is poorly immunogenic in humans (18). Development of vaccines against group B meningococcal disease has focused on the use of lipo-oligosaccharide (LOS)-depleted outer membrane proteins (OMPs) (2, 3). Between 1989 and 1990 an OMP vaccine produced in Cuba was used to immunize 2.4 million children ranging from 3 months to 6 years of age in the city of São Paulo, Brazil. Results of a case control study performed from June 1990 to June 1991 (12 months) showed that vaccine efficacy was age dependent. In children aged 24 to 48 months and aged over 48 months, estimated efficacies were 47 and 74%, respectively. There was no vaccine efficacy in children aged up to 23 months (14). In spite of being statistically significant, levels of protection observed in children 24 months or older were far from ideal and did not have a significant impact on public health as the incidence of the disease was not significantly reduced in São Paulo (14). Also, the duration of the protection induced by the vaccine remains unknown.

Several factors may account for the performance of this OMP vaccine in Brazil. The fact that only a portion (~44%) of the bacterial isolates from infected individuals matched the vaccine type strain (B:4:P1.15) could be a factor that reduced its efficacy (14). An analysis of the presence of bactericidal antibodies in the sera of the vaccinated children found that only 40% had bactericidal antibodies to a B:4:P1:1.15 strain (13). As bactericidal antibodies are believed to be important for the immunity of vaccinated individuals (5), the fact that this vaccine failed to elicit bactericidal antibodies in the majority of children may account for its poor performance. In agreement with this possibility is the fact that a correlation between vaccine efficacy and the increasing prevalence of induced bactericidal antibodies with age was found (13).

Among the five main classes of proteins found in the outer membrane vesicles (OMVs) (classes 1 through 5), PorA protein and class 5 proteins have been suggested to be of great importance for the induction of bactericidal antibodies after immunization and disease (11, 19, 26). In a recent study (25), the specificity of bactericidal antibodies of individuals vaccinated with hexavalent meningococcal PorA protein vesicle vaccine was evaluated by using isogenic strains differing only in their PorA protein compositions. This study demonstrated that the epitopes that contributed predominantly to the bactericidal activity were present in loops 1 and 4 of PorA protein, which contain variant region 1 (VR1) and VR2, respectively. In a parallel study, Rosenqvist et al. (19) demonstrated that PorA protein and class 5 proteins are the major targets of bactericidal antibodies of individuals vaccinated twice with an OMV vaccine.

The present study was designed to evaluate the specificity of bactericidal antibodies from Brazilian children vaccinated with the Cuban OMP vaccine. For that purpose we determined the bactericidal activities of serum samples from selected individ-

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TABLE 1. Serotype, serosubtype, and P5 type of serogroup B meningococcal strains

Strain	Serotype	Serosubtype	P5 type
N150/88	4	P1.15	−[a]
N44/89	4	P1.15	5,7
N43/90	4	P1.15	5,7
N163/90	4	P1.15	5,7
N288/91	4	P1.15	5,7
N20/95	4	P1.15	5,7
N1230/95	4	P1.15	c
N171/96	4	P1.15	5,7
N738/96	4	P1.15	c
N447/96	8,10	P1.15	c
N577/89	4	P1.nt	nt
N585/94	4	P1.nt	c
N433/95	4	P1.nt	nt
N405/96	4	P1.nt	nt
N493/96	4	P1.nt	nt
N539/96	4	P1.nt	c
N654/96	4	P1.nt	c
N292/91	4	P1.16	nt
N1098/93	4	P1.7,1	5,7,c
N7/94	4	P1.3	5,c
N1206/95	8,10	P1.9	nt
N594/96	4	−	5,7
M1.2	4	−	−
R43	4	P1.15	−
Cu385/83[b]	4	P1.15	5,c

[a] −, mutant.
[b] Cu385/83 was isolated in Cuba.

uals against local strains as well as against mutant strains lacking either class 1 or class 5 proteins or both.

MATERIALS AND METHODS

Meningococcal strains. This study included 23 meningococcal strains isolated from clinical cases in São Paulo State. Table 1 shows the phenotypic characteristics of these strains. One strain isolated in Cuba and kindly provided by V. G. G. Sierra was included in the analysis. A variant meningococcal strain lacking PorA protein and class 5 OMP (M1.2) was obtained from strain N44/89 as described by J. Tommassen et al. (24), except that rabbit serum instead of guinea pig serum was used as the complement source. Monoclonal antibody (MAb) F87A2/1H11, which recognizes the P1.15 epitope, was produced at Instituto Adolfo Lutz. A variant of strain N44/89 lacking the class 5 OMP (strain R43) was recovered after serial cultures on Mueller-Hinton agar (Difco) (21).

Serum samples from vaccinees. Blood samples were collected from children 2 to 6 years old during an immunization campaign conducted in greater São Paulo in 1989 in which the Cuban serogroup BC vaccine was used (13). Blood samples were drawn before the first dose and 4 weeks after the second vaccination. Serum samples were stored at −20°C. The numbers 1 to 16 were assigned to each serum sample and maintained throughout the study for their precise identification.

Bactericidal assay. Serum bactericidal antibodies were detected as described by Maslanka et al. (12) with some modifications. Briefly, the final reaction mixture contained 25 μl of dilutions of test sera previously heat inactivated at 56°C for 30 min, 12.5 μl of human serum as the complement source, and 12.5 μl of log-phase meningococci (about 3×10^4 CFU/ml) grown in tryptic soy agar (Difco). The reaction was carried out at 37°C for 30 min. The bactericidal titer was defined as the reciprocal of the serum dilution yielding ≥50% killing of bacteria. A positive control was included in each plate. The complement-independent killing control consisted of heat-inactivated unknown test sera in the presence of heat-inactivated complement and bacteria. Serum samples were titrated only against the N44/89 strain. To determine the serum bactericidal activity against the other meningococcal strains (Table 1), the last dilution and the one just before, which resulted in more than 50% killing of the N44/89 strain, were employed. Strain Cu385/83 was grown in tryptic soy agar (Difco) with sufficient iron or under conditions of iron limitation when it was used as a target strain. Ethylenediamine di-*o*-hydroxyphenylacetic acid (Sigma) at 45 mM was used to chelate free ferric iron and induce the formation of iron-regulated proteins (IRPs) in the outer membrane.

Extraction of OMVs and SDS-PAGE and immunoblotting. These procedures were done as described previously (13). To determine the LOS profiles of strains studied, OMVs were electrophoresed through 15% acrylamide gel by using a tricine–sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system. LOS components were visualized by silver staining (10). Strains 6940 (L1.8) and Cu385/83 (L3,7,9) were used as the controls in each gel.

Class 5 protein typing. The class 5 proteins of all strains used in this study were characterized by SDS-PAGE analysis and immunoblotting. MAbs P5.5 (3DH3-F5GE), P5.7 (AG10), P5.8 (F1-5D5/1D2), and P5.c (279-5c) were used in the immunoblotting reactions.

DNA preparation. Genomic DNA was obtained as earlier described (1). The DNA concentration was estimated by comparison with standard DNA (lambda *Hin*dIII-digested DNA; Life Technologies Gibco BRL, Gaithersburg, Md.) on a 1% agarose gel.

PCR and DNA hybridization. PCR amplifications of the class 1 genes of meningococcal strains were performed with primers CH1 (5′-CGTATCGGGT GTTTGCCCGA-3′) and CH2 (5′-TTAGAATTTGTGGCGCAAACC-3′) purchased from Oligos Etc. Inc., Wilsonville, Oreg.). PCR components were as follows: 1 μg of template (meningococcal chromosomal DNA); 10 mM Tris-HCl (pH 8.0); 50 mM KCl; 1.5 mM $MgCl_2$; 200 μM (each) dATP, dCTP, dGTP, and dTTP; 0.5 U of *Taq* DNA polymerase (Perkin-Elmer, Branchburg, N.J.); and 100 ng of each primer. Reaction mixtures were first incubated for 5 min at 94°C. Then, 35 cycles were performed as follows: 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C. The products of PCR were analyzed on a 1% agarose gel in 40 mM Tris-acetate–1 mM EDTA (pH 8.0) at 50 to 100 mA. For Southern hybridization, agarose gels were blotted onto nylon membrane filters (Amersham, Cleveland, Ohio). The filters were fixed by irradiation with UV light with a 254-nm wavelength for 3 min. Oligonucleotide probes for VR1, 5′-CCGCCCTCAAAGAGT CAACCTCAG-3′, and VR2, 5′-GCTCATTATACTAGGCAGAACAAT-3′, were purchased from Oligos Etc. Inc. Oligonucleotides were labeled with [γ-^{32}P] ATP (Amersham) by using T4 polynucleotide kinase (Life Technologies Gibco BRL) as described by the manufacturer's procedure and were purified on a Sephadex G50 (Sigma, St. Louis, Mo.) gel filtration column as described in reference 23. Prehybridization was performed for 2 h in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) buffer–0.1% SDS–1 mM EDTA–10 mM NaH_2PO_4–0.2% milk at 42°C. For hybridization, radiolabeled probe (~10^6 cpm/ml) was heated to 100°C for 2 min and added to the prehybridization mixture and the mixture was incubated with rotation overnight at 42°C. The filters were washed twice for 15 min in 6× SSC buffer with 0.1% SDS at 42°C, followed by one washing in 1× SSC buffer with 0.1% SDS as described before. Filters were then autoradiographed at room temperature with an intensifying screen on Kodak film. The P1.15 PorA gene was obtained by cloning the PCR product obtained by amplification from DNA isolated from strain N44/89 into pMOS Bluescript vector (Amersham) according to the protocol suggested by the manufacturer. The insert was partially sequenced to insure that the gene cloned was indeed the P1.15 PorA gene. The gene was excised from the vector and labeled with [α-^{32}P] dCTP (Amersham) with the aid of a random primer labeling kit (Gibco BRL Life Technologies). The probe was separated from the free isotope as described above and used for hybridization. Prehybridization, hybridization, washes, and autoradiography were performed as described above.

PorA gene sequencing. Nucleotide sequence analysis of PCR products of strains N738/96 and N43/90 was done with the *Taq* Dye-Deoxy terminator cycle sequencing kit (Applied Biosystems, Foster City, Calif.) according to the manufacturer's instructions. For sequencing the PorA gene encoding the class 1 protein, 14 primers (7 forward and 7 reverse), designed to be complementary to the conserved regions of the *por*A gene, were used. Details of the sequences of the primers will be published elsewhere (20a). Sequencing products were purified by using Centri-Sep spin columns (Princeton Separations, Adelphia, N.J.) and were run an ABI model 373S automated DNA sequencing apparatus. Full-length sequences were translated to the amino acid sequences, which were then aligned.

Nucleotide sequence accession numbers. The GenBank accession numbers for the sequences identified in this study are AF012011 and AF012012.

RESULTS

Antibodies bactericidal for meningococci isolated in Brazil. To evaluate the specificity patterns of bactericidal antibodies elicited by immunization with OMPs, pre- and postvaccination serum samples from 77 children immunized with the Cuban vaccine were titrated for the presence of bactericidal antibodies against strain N44/89. Twenty vaccinees (26%) had at least a fourfold increase in bactericidal titers after vaccination. The $\log_2$'s of the mean titers before and after vaccination were 1.26 and 3.5, respectively ($P < 0.05$) (data not shown). Sixteen samples were selected for our bactericidal assays on the basis of the volume available. Prevaccination serum samples showed no significant bactericidal activity to all strains studied, except for serum from individuals 2 and 4, which lysed strain N1230/95.

Earlier studies have suggested that PorA protein is an important target for bactericidal antibodies (19, 24, 26). There-

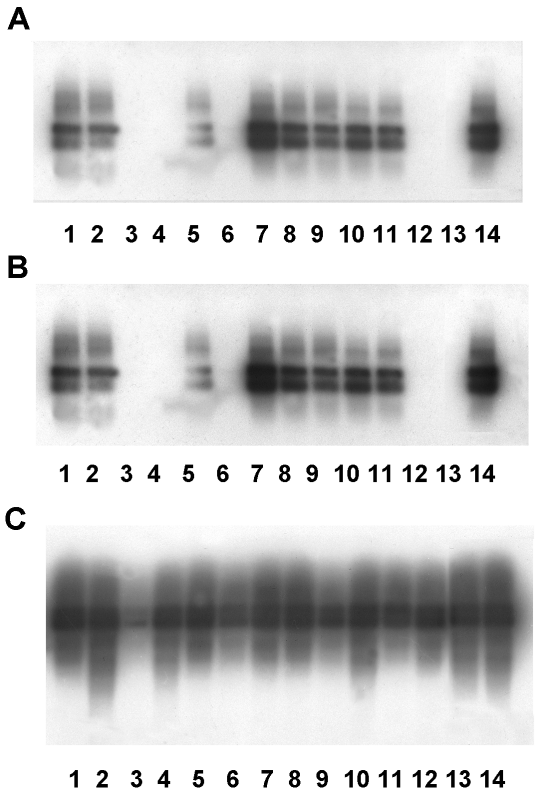

FIG. 1. Southern hybridization analysis of PCR-amplified PorA gene probed with the ^{32}P-labeled coding sequences for VR1 (A) and VR2 (B) of strain N44/89 and with the ^{32}P-labeled PorA gene of strain N44/89 (C). Strains in lanes 1 to 14: N20/95, N1230/95, N1098/93, N7/94, N447/96, N1206/95, N150/88, N171/96, N163/90, N43/90, N288/91, N292/91, N337/89 (negative control), and N44/89 (positive control), respectively.

fore, our first intention was to determine whether serum from 14 vaccinees would lyse a panel of nine Brazilian isolates containing PorA proteins homologous to that of the vaccine strain. A set of sera was also tested with the Cuban vaccine strain (Cu385/83). The Brazilian strains were initially identified as B:4:P1.15 by conventional methods using MAbs, and they were isolated predominantly in São Paulo from 1988 through 1996 (see details in Table 1). To confirm their serosubtypes, we used oligonucleotide probes containing sequences representing the VR1- and VR2-coding regions of the P1:15 gene. As depicted in Fig. 1A and B (lanes 1, 2, 5, 7–11, and 14) DNA isolated from all strains contains sequences that hybridize with both variable regions of the P1.15 gene. Figure 1C shows the hybridization with a DNA probe containing the entire PorA gene. In spite of the fact that all isolates belong to serosubtype P1.15, not a single serum sample had bactericidal antibodies against all strains (Table 2). Only in few cases could a clear pattern of recognition be correlated with a certain bacterial strain. Two strains, N447/96 and N171/96, were recognized by bactericidal antibodies present in all serum samples tested. In contrast, strain N738/96 could not be lysed by any of the 14 serum samples tested and only 2 of 9 samples had bactericidal antibodies against strain N43/90. To determine whether the lack of recognition of these two strains was due to mutations, PorA genes of these two strains were entirely sequenced. We did not find a single base pair difference among these genes and those of strains N44/89 and Cu385/83 (data not shown). We concluded from these experiments that bactericidal antibodies elicited by immunization with the Cuban OMP-based vaccine cannot lyse all B:4:P1:15 isolates prevalent in Brazil (22). Moreover the B:4:P1.15 strain (Cu385/83) isolated in Cuba was killed by only 50% of sera tested (Table 2).

Considering the fact that the vaccine used in this study contains a complex of high-molecular-weight proteins, we decided to analyze the bactericidal antibody response to the Cuban strain either expressing or not expressing IRPs. The antibody responses of the seven sera tested were the same regardless of whether the target strain contained IRPs or not (data not shown).

Subsequently, we evaluated whether these vaccinees had bactericidal antibodies to heterologous serosubtype strains. A representative strain for each of the main serosubtypes isolated in Brazil was used: P1.7,1, P1.9, P1.3, and P1.16. As shown in Fig. 2, 50% of the vaccinees (individuals 1, 2, 6, 8, 9, 12, and 13) had bactericidal antibodies against at least one of the three heterologous strains. However, it is noteworthy that none of the vaccinees had antibodies to the P1.16 strain.

Taking into account the significant proportion of nonserosubtypeable isolates in Brazil, a set of P1.nt strains was also included in this study. A quite similar pattern was observed when we examined for the presence of bactericidal antibodies to P1.nt strains. As shown in Table 3, bactericidal antibodies against at least one of the seven strains were detected in 9 of the 14 vaccinees (individuals 1, 2, 4, 6, 8, 9, 12, 13, and 14). To

TABLE 2. Bactericidal activities of postvaccination serum samples against P1.15 meningococcal strains

Individual	Activity[a] of serum against strain:									
	N150/88	N43/90	N163/90	N288/91	N20/95	N1230/95	N171/96	N738/96	N447/96	Cu385/83
1	−	+	+	+	+	+	+	−	+	−
2					+	+		−	+	
3	+	−	+	+	−	+	+	−	+	+
4	+	−	+	+	−	+	+	−	+	+
5	+	−	+	+	−	+	+	−	+	+
6	+	−		+	+	−	+	−	+	−
7	+	−	+	+	−	+	+	−	+	+
8					+	+		−	+	−
9					+	+		−	+	
10	+	−	+	+	−	+	+	−	+	+
11					−	−		−	+	−
12	+	+	−	+	+	−	+	−	+	+
13		−	−	−	+	−		−	+	−
14					+	+		−	+	

[a] Blank spaces indicate that the serum was not tested. +, ≥50% killing; <50% killing.

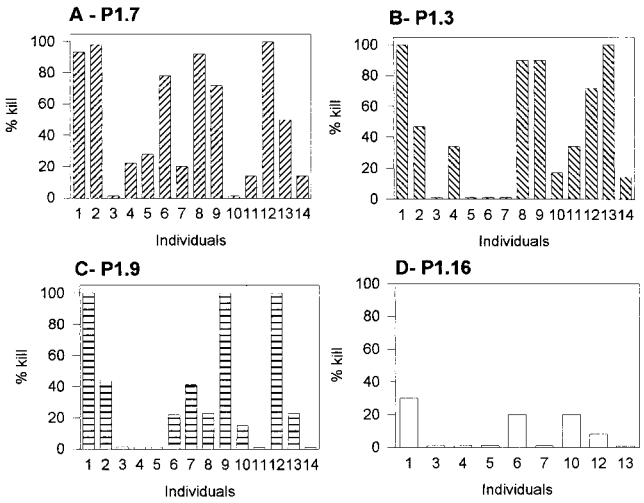

FIG. 2. Bactericidal activities (% kill) of 14 postvaccination serum samples from children 2 to 6 years old against the indicated heterologous serosubtypes.

ensure that the PorA genes of all heterologous strains used in the bactericidal assay did not contain sequences encoding VR1 and VR2 of P1.15, we performed a Southern blot hybridization with oligonucleotide probes representing these sequences. The DNA segments corresponding to the PorA genes of these strains failed to hybridize with these two oligonucleotide probes (Fig. 1A and B and 3A and B). In contrast, they hybridized with a DNA probe containing the entire PorA gene used as the positive control (Fig. 1C and 3C). These results demonstrate that more than half of the vaccinees have bactericidal antibodies that recognize cross-reactive epitopes present in two or more natural isolates distinct from subtype P1.15. These antibodies are not specific for VR1 and VR2 of the P1:15 protein. Also, they do not recognize epitopes conserved among all strains because none of the serum samples lysed all isolates.

Bactericidal antibodies to class 1 and class 5 mutants. To determine whether these individuals had postvaccination antibodies to either class 1 or class 5 proteins or both, we used laboratory-generated and natural mutants. One of these mutants (strain M1.2) was isolated in our laboratory and failed to express class 1 and class 5 proteins as determined by SDS-PAGE and immunoblotting using specific MAbs. This mutant expresses normal amounts of class 3 protein as determined by SDS-PAGE and by using a MAb specific for serotype 4 (data not shown). Although all 16 sera were bactericidal for the original N44/89 strain, the reactivities of 12 samples (75%) dropped significantly, demonstrating that these two proteins are the main targets of the bactericidal antibodies present in the sera of these individuals (Fig. 4). It is, however, important to notice that a significant proportion (25%) of individuals have bactericidal antibodies to an OMV component(s) other than the class 1 and class 5 proteins.

To ascertain whether bactericidal antibodies were directed against the class 1 or class 5 proteins, the 12 sera nonbacteri-

TABLE 3. Bactericidal activities of postvaccination serum samples against B:4:nt meningococcal strains

Individual	Activity[a] of serum against strain:						
	N577/89	N585/94	N433/95	N405/96	N493/96	N539/96	N654/96
1	−	−	−	+	+	−	−
2	−	+	−	−	+	+	+
3	−	−	−	−	−	−	−
4	−	−	−	−	−	+	+
5	−	−	−	−	−	−	−
6	+	+	−	−	−	−	−
7	−	−	−	−	−	−	−
8	+	+	+	−	+	−	+
9	−	−	−	−	+	−	−
10	−	−	−	−	−	−	−
11	−	−	−	−	−	−	−
12	+	−	+	−	−	−	−
13	+	+	+	−	+	−	+
14	−	+	+	−	+	−	+

[a] +, ≥50% killing; −, <50% killing.

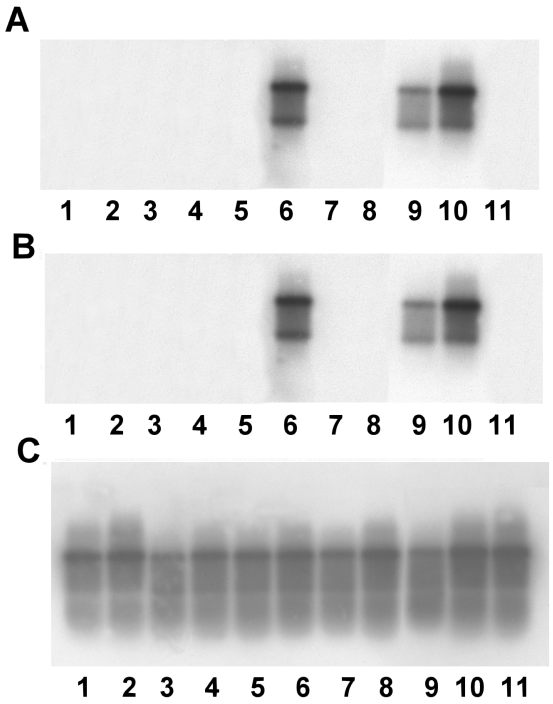

FIG. 3. Southern hybridization analysis of PCR-amplified PorA gene probed with ^{32}P-labeled coding sequences for VR1 (A) and VR2 (B) of strain N44/89 and with the ^{32}P-labeled PorA gene of strain N44/89 (C). Strains in lanes 1 to 11: N577/89, N585/94, N405/96, N493/96, N539/96, N594/96, N654/96, N433/95, N738/96, N44/89 (positive control), and N337/89 (negative control), respectively.

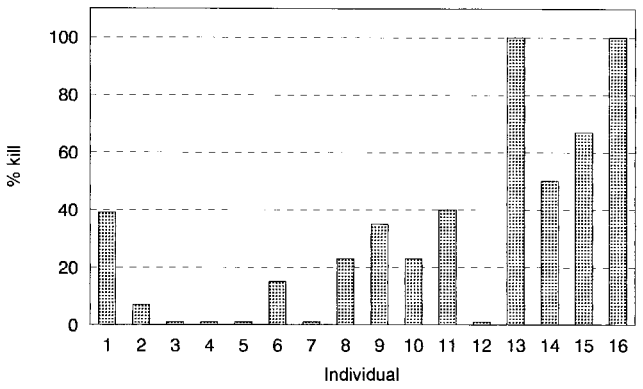

FIG. 4. Bactericidal activities (% kill) of 16 postvaccination serum samples from children 4 to 6 years old against a variant of strain N44/89 lacking the PorA protein and OMP5 (strain M1.2).

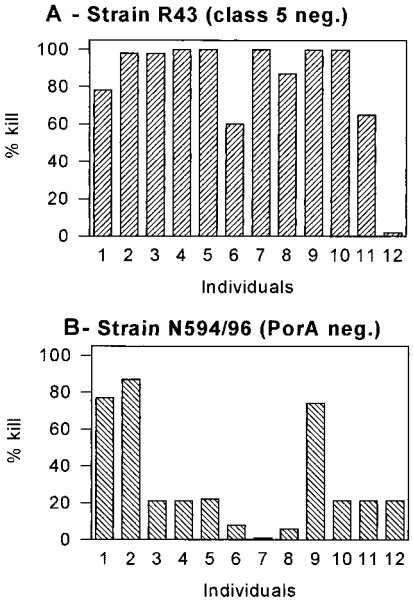

FIG. 5. Bactericidal activities (% kill) of 12 postvaccination serum samples from children 2 to 6 years old against a variant of strain N44/89 lacking the class 5 protein (strain R43) (A) and a natural PorA protein-negative isolate (strain N594/96) (B).

cidal for mutant M1.2 were examined for bactericidal killing of two mutants lacking either a class 1 or class 5 protein. The class 5 protein-negative mutant (strain R43) originated in the laboratory from strain N44/89 and expresses normal levels of class 1 and class 3 proteins. The class 1 protein-negative mutant (N594/96) was isolated in São Paulo and expresses normal levels of class 3 protein of serotype 4 and class 5 protein of the same antigenic specificity as that of the class 5 protein of the N44/89 strain (P5.5,7) (Table 1). Bactericidal antibodies to the class 5 protein-negative mutant could be detected in 11 of 12 serum samples (Fig. 5a). The fact that these sera failed to lyse a class 1 protein- and class 5 protein-negative double mutant, indicates that they have bactericidal antibodies against the class 1 protein. In contrast, serum antibodies of individual number 12 failed completely to lyse the class 5 protein-negative mutant, and therefore his bactericidal antibodies seem to be directed solely against the class 5 protein. Bactericidal antibodies against N594/96 (class 1 protein-negative mutant) were detected in 3 of 12 serum samples (individuals 1, 2, and 9) (Fig. 5B). The fact that this mutant was not generated from strain N44/89 restricts clear-cut interpretations. However, it is very likely that these three individuals have bactericidal antibodies that recognize class 1 and 5 proteins.

DISCUSSION

Results from our studies indicated that class 1 and class 5 proteins are the main targets recognized by bactericidal antibodies from Brazilian children immunized with the Cuban OMP vaccine. Serum from 12 of 16 individuals failed to present bactericidal antibodies to a double-mutant strain lacking class 1 and class 5 proteins. These results are in full agreement with previous reports that have implicated these two antigens as important targets for bactericidal antibodies induced by vaccination (19, 25, 26). Nevertheless, sera from four individuals had strong bactericidal activity against the double mutant, suggesting that antibodies of a important fraction of the population may recognize an as yet unidentified target. Our results are in agreement with a recent study that found that a large fraction (68%) of Norwegian adult vaccinees who received three doses of the OMV vaccine of strain 44/76 had bactericidal antibodies to a mutant strain lacking both class 1 and 5c proteins (19).

Sera from a group of individuals (group 1) seem to present bactericidal antibodies only to the class 1 protein. This group is composed of individuals 3, 5, 7, 10, and 11. Their antibodies failed to lyse both class 1 mutant strains and were capable of killing only bacterial strains with the P1.15 serosubtype. Therefore, we concluded that a certain percentage of vaccinees develop bactericidal antibodies only to the class 1 protein. It is noteworthy, however, that 3 of 10 isolates (N738/96, N43/90, and N20/95) were not lysed by the sera of these five individuals. Sera from vaccinees other than this group were capable of lysing two of these strains (N43/90 and N20/95). The fact that some of the P1.15 isolates are not recognized by bactericidal antibodies can be explained by several not mutually exclusive reasons. First, it is possible that a mutation in the PorA gene that impairs class 1 recognition had occurred. However, this was not the case since a sequence analysis of the PorA genes of strains N738/96 and N43/90 did not reveal any difference compared to the sequences of the PorA genes of strain N44/89 and strain Cu385/83 and those of other PorA genes described. It can also be explained by low levels of expression of the PorA protein. SDS-PAGE analysis of OMV showed that all three strains not lysed by antibodies from individuals in group 1 expressed a reduced amount of PorA protein (data not shown). However, other differences may contribute to the resistance to killing of these isolates since strain N1230/95, despite having little PorA protein, was killed by antibodies from all but one individual from that group. Finally, the immunotype and length of the LOS could affect bactericidal resistance. Expression of the L8 LOS immunotype is correlated with increased sensitivity to serum bactericidal activity (15). However, based on tricine–SDS-PAGE followed by silver stain analysis, we have determined that all strains have a LOS profile similar to that of a L3,7,9 strain (Cu385/83), in which one or two bands of low molecular weight are presented. The LOS band of strain 6940 (L1.8) had a molecular weight intermediate to those of the two bands of L3,7,9 LOS (data not shown).

Another group of vaccinees have bactericidal antibodies that recognize cross-reactive epitopes present in a variety of strains distinct from B:4:P1.15. Individuals 1, 2, and 9 have bactericidal antibodies to both class 1 and class 5 proteins. Their sera killed both single mutants but not the double mutant. Sera from this group have an extensive cross-reactivity with several other bacterial strains that can be explained, at least in part, by the recognition of class 5 proteins. Immunoglobulin G binding to class 5 proteins of P1.7 and P1.3 strains, which have partial homology to the class 5 protein of strain N44/89 (Table 1), was detected by immunoblot studies (data not shown). However, we cannot completely rule out the possibility that these individuals have cross-reactive bactericidal antibodies against conserved regions of class 1 as well as class 5 proteins. Other studies have shown the antibody responses of patients convalescent from meningococcal disease and of vaccinees to PorA protein epitopes different from those defined by the subtyping MAbs (7, 8, 16). Individuals 6 and 8 have a pattern of recognition that includes antibodies to class 1 proteins as well as antibodies to cross-reactive epitopes. We could not find a definitive proof that they have antibodies to class 5 proteins, as their sera did not lyse the class 1 protein-negative single mutant. However, the fact that our class 1 protein-negative mu-

tant was not generated from strain N44/89 may explain the absence of bactericidal antibodies.

Rosenqvist et al. (20) showed that antibodies to 5c protein contribute significantly to the bactericidal activity detected in Norwegian vaccines. Besides, human MAbs to 5c isolated from a individual immunized with the Cuban vaccine have been described previously (4). Individual 4 differed from other individuals from group 1 because his serum recognized two P1.nt strains (N539/99 and N654/96) that express a significant amount of 5c protein (data not shown). Therefore, further analyses of antibody specificity should include the antibody response to the 5c protein.

The fact that bactericidal antibodies from different individuals recognize distinct OMPs has been noted in earlier studies (11, 26). This complex pattern of recognition by bactericidal antibodies of Brazilian vaccinees has important implications for understanding the outcome of previous and future vaccination trials performed in Brazil. During the past trials, the protection elicited by the Cuban vaccine was partial. Several explanations may account for this outcome. Our results can explain, at least in part, why the levels of protection found were not greater. According to our data none of the vaccinated children have bactericidal antibodies capable of killing all wild-type isolates tested; therefore, none of them can be considered completely immune to *Neisseria meningiditis* infection. Also, our results suggest that individuals with bactericidal antibodies to cross-reactive epitopes present in several serosubtypes may have a better chance of being protected than individuals with bactericidal antibodies restricted to the PorA proteins as in the case of previous described vaccines (25). This hypothesis will have to be clinically confirmed in future studies. Also, the involvement of bactericidal target antigens distinct from the PorA protein has been suggested by a study of human antibody response after disease or asymptomatic carriage (9).

Another study of convalescent-phase serum from patients with meningococcal disease showed a higher-level immunoglobulin G response against the class 3 protein (PorB protein) than against the PorA protein (6). All but two strains of this study belong to serotype 4. The levels of bactericidal antibodies against the double-mutant strain suggest that most of the vaccinees had no bactericidal antibodies against PorB. Besides, two other B:4 strains (N292/91 and N738/96) could not be killed by any serum tested.

In summary, our study and those of others highlight the importance of determining the fine epitope specificity of bactericidal antibodies to improve the existing vaccines based on OMPs or OMVs. Knowledge about the specificity patterns of functional antibodies induced by immunization or disease may be important for the definition of protective antibodies. Also, it may be useful for designing a vaccine that induces bactericidal antibodies against a broad range of group B organisms.

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