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Immune Response of Brazilian Children to a *Neisseria meningitidis* Serogroup B Outer Membrane Protein Vaccine: Comparison with Efficacy

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Since 1986, serogroup B *Neisseria meningitidis* has caused approximately 80% of the meningococcal disease in Brazil. In 1988, an epidemic caused by *N. meningitidis* B:4:P1.15 was recognized in the greater São Paulo area of Brazil. The São Paulo state government decided to vaccinate children from 3 to 83 months of age with a vaccine consisting of serotype 4 outer membrane protein and group C meningococcal polysaccharide that was produced in Cuba. About 2.7 million children were vaccinated during two immunization campaigns conducted in 1989 and 1990. Because of this, a case-control study was designed to determine vaccine efficacy against group B meningococcal disease. The purpose of our study was to compare the antibody response with the protection from disease estimated from the case-control study. We measured the immune responses of vaccinees by enzyme-linked immunosorbent assay (ELISA), immunoblot, and bactericidal assay. The development of bactericidal antibodies was age dependent and in good agreement with the results of the case-control study. Only 40% of vaccinees showed fourfold or greater increases in bactericidal antibody titers after vaccination. A poor correlation between antibody levels detected by ELISA and those by bactericidal assay was found. Immunoblot analysis showed that about 50% of the serum samples with bactericidal titers higher than 1:4 were reactive with class 1 outer membrane protein. We conclude that the bactericidal assay is a good, laboratory-based, functional assay for the study of vaccine immunogenicity and that an effective solution to group B meningococcal disease remains to be demonstrated.

Since 1986, serogroup B *Neisseria meningitidis* has been responsible for approximately 80% of the meningococcal disease in Brazil (29). In 1988, an epidemic situation in the greater São Paulo area of Brazil, which extends up to the present, was recognized (6). In 1990, greater São Paulo included 17.8 million inhabitants in 38 municipal regions. A single serogroup B *N. meningitidis* clone, being of the ET-5 complex and having serotype 4:P1.15, has been the prevalent strain in the region (8, 29).

Effective polysaccharide vaccines against *N. meningitidis* serogroups A, C, Y, and W135 are available (10, 15, 17). In contrast, the serogroup B polysaccharide is poorly immunogenic and antibodies to this polysaccharide do not appear to be protective (21). Most of the efforts to develop an effective serogroup B vaccine have therefore focused on lipooligosaccharide-depleted outer membrane proteins (OMPs) (2, 14, 27, 36). Efficacy trials with such vaccines have recently been conducted in Chile, Cuba, and Norway (3, 5, 32). Efficacy levels of approximately 50% were found in the Chile and Norway studies. Better protection was reported for the vaccine produced in Cuba, with an efficacy around 80% against disease caused by a B:4:P1.15 strain (32). This was the first clear demonstration that antibodies induced to noncapsular antigens can protect against meningococcal disease. In view of the epidemic situation in greater São Paulo, i.e., 4.06 cases per 100,000 inhabitants in 1988, and recent acquisition of the Cuban serogroup BC vaccine by the São Paulo state government, it was decided to vaccinate all children from 3 months to 7 years of age. Two immunization campaigns were conducted. In the first (1989), about 300,000 children attending day care centers were vaccinated (12% of the estimated 2.7 million children in the target age range), and in the second (1990), about 2.4 million children (92% of children in the target age range) were vaccinated. Both campaigns were carried out in regional health clinics where children received two doses of the vaccine (6, 7). The vaccination campaigns in São Paulo were the first use of the Cuban-produced vaccine outside of Cuba. Analysis of the immune responses of vaccinated children was considered fundamental to an evaluation of vaccine effectiveness in Brazil. The purpose of this study was to measure the antibody responses of vaccinees by quantitative and functional methods such as enzyme-linked immunosorbent assay (ELISA) and bactericidal assay and to correlate these responses with the protection against disease by using the results of the case-control study (24), which was designed to determine vaccine efficacy against group B meningococcal disease.

MATERIALS AND METHODS

Vaccine. The vaccine was produced in Cuba (it is often referred to as Cuban BC vaccine) from strain Cu385/83 (B:4:P1.15), consisted of lipooligosaccharide-depleted OMPs and group C polysaccharide, and was enriched with envelope proteins from 65 to 95 kDa (32). The group C polysaccharide was present to improve the solubility of the OMPs and to provide protection against group C meningococcal disease. The vaccine was adsorbed onto aluminum hydroxide. Each dose contains 50 μg of group B proteins, 50 μg of C polysac-

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TABLE 1. Antibody levels as measured by ELISA pre- and postvaccination with the Cuban BC vaccine

Age group (mo)	No. of children	Strain of ELISA antigen	Antibody levels (U/ml)[a]		Fold increase	% >Twofold[b]	% >Fourfold[b]
			Prevaccination	Postvaccination			
3–23	275	Cu385/83	25.5	111.2	4.4	81	51
	276	N.150/88	21.7	59.0	2.7	63	35
	120	N.44/89	36.1	156.7	4.3	84	56
	120	N.577/89	37.2	136.3	3.7	85	51
	120	N.614/89	23.9	71.0	3.0	80	42
24–47	144	Cu385/83	23.3	110.5	4.8	85	54
	144	N.150/88	18.6	73.9	4.0	76	45
	20	N.44/89	20.8	97.0	4.7	100	60
	21	N.577/89	20.9	83.9	4.0	90	52
	21	N.614/89	17.0	53.6	3.2	95	38
48–83	141	Cu385/83	27.1	148.0	5.5	87	56
	141	N.150/88	17.6	82.4	4.7	86	56
	19	N.44/89	31.1	173.1	5.6	95	58
	19	N.577/89	23.9	108.9	4.6	89	63
	19	N.614/89	26.3	100.4	3.8	84	58

[a] Geometric mean units per milliliter.
[b] Percentages of vaccinees showing greater than two- and fourfold increases in antibody levels, respectively.

charide, and 2 mg of aluminum hydroxide with 0.01% thimerosal as a preservative (32). Two intramuscular doses of 0.5 ml were administered to children between the ages of 3 and 83 months at an interval of 6 to 8 weeks.

Serum samples from vaccinees. During the first campaign, blood samples were collected before the first dose and 4 weeks after the second vaccination. Serum samples were stored at −20°C. For serological studies, children were classified in the three age groups used for the case-control study. The age groups and numbers of individuals studied can be seen in Tables 1 and 2.

Meningococcal strains. Brazilian strains designated N.44/89 (B:4:P1.15), N.150/88 (B:4:P1.15), N.577/89 (B:4:nt), and N.614/89 (B:NT:P1.15) and a Cuban strain [Cu385/83 (B:4:P1.15)] were used for the preparation of ELISA solid-phase outer membrane antigens. Strains Cu385/83 and N.150/88 were used in immunoblot analysis and as the target strain for the bactericidal assay, respectively. These strains were serogrouped by slide agglutination and serotyped by immunoblot analysis with monoclonal antibodies (29).

ELISA and immunoblot antigens. Cultures of *N. meningitidis* were grown overnight at 36°C in tryptic soy broth (TSB) (Difco Laboratories, Detroit, Mich.) on a rotatory shaker at 120 rpm. Outer membrane vesicles (OMV) were prepared by extraction of the wet cell pellet for 2.5 h at 50°C with 5 ml of 0.2 M lithium chloride in a 0.1 M sodium acetate buffer (pH 5.8) per g of cells (33). Protein concentrations were determined by the method of Lowry et al. (22), and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyze the OMV proteins. Sialic acid and 2-keto-3-deoxyoctulosonic acid were analyzed by the resorcinol and thiobarbituric acid techniques, respectively (26, 34). Additionally, strain Cu385/83 was grown in TSB containing 42 μM EDDA [ethylenediamine di(*o*-hydroxyphenylacetic acid); Sigma Chemical Company, St. Louis, Mo.] to chelate free ferric iron and induce the formation of iron-regulated proteins (IRPs). OMVs containing IRPs were used only for immunoblot analysis.

ELISA technique. A standardized ELISA was performed in triplicate in microdilution plates (Difco) as described by Harthug et al. (19) with an alkaline phosphatase-conjugated goat anti-human immunoglobulin G (IgG) (Sigma) detection system. As an internal antibody standard, a twofold dilution series of a positive postvaccination serum sample was used in all experiments. The mean value of the observed optical density was transformed to arbitrary units per milliliter by a sigmoidal standard curve (log-logit transformation) calculated from the values of the reference serum sample with a computer program provided by Carl E. Frasch. Initially, all serum samples were analyzed at a 1:200 dilution. Samples with optical density values of ≥90% of the maximum optical density of the standard were further diluted and reanalyzed.

Bactericidal assay. A modification of the assay described by Frasch and Robbins was used for determining bactericidal activity (12). Serum samples were titrated in flat-bottom plates (Linbro; Flow) with Hanks' balanced salt solution containing

TABLE 2. Geometric mean bactericidal titers against *N. meningitidis* N.150/88 (B:4:P1.15) in serum samples from vaccinees

Age group (mo)	No. of children	GMT[a]		% >1:4[b]		% ≥Fourfold increase[c]	% Point estimate of vaccine efficacy (95% CI)[d]
		Prevaccination	Postvaccination	Prevaccination	Postvaccination		
3–23	122	1.1	2.0	2.5	13.1	22	−37 (−100 to 73%)
24–47	44	1.5	4.9	20.4	43.2	45	47 (−72 to 84%)
48–83	44	2.0	9.2	25.0	52.3	52	74 (16 to 92%)
Total	210	1.5	4.6	10.9	36.2	40	54 (Not given)

[a] GMT, geometric mean bactericidal titer.
[b] Percentages of vaccinees with bactericidal titers of greater than 1:4.
[c] Percentages of vaccinees with bactericidal titer increases of at least fourfold.
[d] From the case-control study (24), with the 95% CI also given.

0.1% bovine serum albumin (HBSS-BSA) as diluent. Meningococci were grown to log phase (2 h) in Mueller-Hinton broth (Difco), and then an equal volume of 10% milk (Oxoid) was added. The culture was then aliquoted and frozen at −70°C. An aliquot was thawed and diluted to yield approximately 2,000 CFU/ml, with the final dilution in HBSS-BSA. By this method, a uniform number of organisms could be used for each assay and a comparison with freshly grown cells gave nearly identical results. The source of complement was human serum from a single donor that lacked bactericidal activity against the target strain and by immunoblot showed no reactivity against the class 1 through class 5 OMPs of strain N.44/89. Microtitration plates were incubated at 37°C for 30 min, and approximately 150 μl of Mueller-Hinton agar containing 10% horse serum and VCN inhibitor (BBL Microbiological Systems, Cockeysville, Md.) was added to each well. Plates were further incubated for 24 h at 37°C with 5% CO_2. The CFU per well were determined with the aid of a stereoscopic microscope (×40). The bactericidal titer was determined with the reciprocal serum dilution yielding a ≥50% reduction in the viable count. A positive serum sample and bacterial inoculum plus complement source controls were included in each experiment.

Immunoblot. SDS-PAGE and the detection of antibodies by immunoblot were performed as described by Wedege and Frøholm (35), except that Tris-glycine transfer buffer without methanol was used. Monoclonal antibodies against type 4 (2303C5), subtype P1.15 (2731C6), and class 4 (AE3) and class 5 (AG10) OMPs were used to identify the class 1 to 5 proteins. The monoclonal antibodies 2303C5 and 2731C6 were produced by one of us (C.E.F.), and the monoclonal antibodies AE3 and AG10 were provided by Biomanguinhos Institute, Rio de Janeiro, Brazil. For class 3 monoclonal antibody reaction, Empigen BB 0.25% (Albright and Wilson, Whitehaven, United Kingdom) was added to the primary antibody solution (23). The binding of human IgG and mouse IgG was detected with goat anti-human and anti-mouse IgG antibodies conjugated with horseradish peroxidase. The pre- and postvaccination serum samples of 15 individuals with postvaccination titers greater than 1:4 were tested.

Statistical methods. The ELISA and bactericidal assay results were transformed to logarithmic values to calculate the geometric means. This gave a normal distribution of data and allowed us to use standard statistical tests (1). The significance levels of differences between groups were examined by the Student *t* test, Wilcoxon scores, or the Kruskal-Wallis test on the log-transformed data. Fisher's exact test was used to analyze the differences among percentages.

RESULTS

Antigenic analysis. SDS-PAGE analysis of the antigens used for ELISA showed a predominance of class 1, 3, 4, and 5 OMPs. In strain N.150/88, class 4 and 5 OMPs were present in small amounts (data not shown).

The antigens used for immunoblot analysis can be seen in the first lane of Fig. 1. These nitrocellulose strips were stained with amido black. Class 1, 3, 4, and 5 OMPs predominated for both antigens. Also, some high-molecular-weight IRPs can be seen with antigens from strains grown in TSB with EDDA (Fig. 1B).

For all antigen preparations, the ratio of protein to lipooligosaccharide was approximately 1:1 and only traces of sialic acid could be detected.

Antibody responses to OMPs. Table 1 shows the ELISA antibody levels against different *N. meningitidis* group B strains

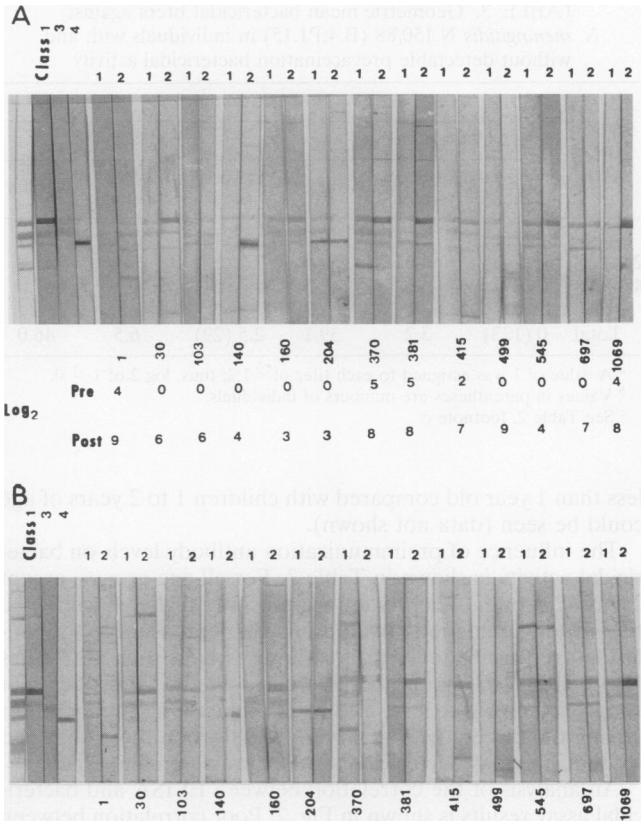

FIG. 1. Immunoblots showing IgG binding to OMPs of *N. meningitidis* Cu385/83 (B:4:P1.15) of pre- (lanes 1) and postimmunization (lanes 2) serum samples from children older than 24 months of age. IgG binding to antigens extracted from the strain grown in normal TSB (A) and TSB containing added EDDA (B) is shown. Amido black-stained strips are shown in the lane at the far left. The next three lanes represent monoclonal antibodies binding to class 1, 3, and 4 proteins, respectively. The bactericidal titers (expressed as $\log_2$ of the reciprocal titers) for pre- and postimmunization serum samples are also shown. The numbers 1, 30, 103, etc., represent vaccinees.

for the age groups examined. The observed differences between the pre- and postimmunization serum samples for all age groups were statistically significant ($P < 0.05$). There were no significant differences in the antibody levels or percent responders among vaccinees among the three age groups studied, except for children from 3 to 23 months of age who showed lower responses against strain N.150/88 ($P < 0.05$). Of all vaccinees studied, 85 and 52% showed at least two- or fourfold increases in antibody levels after vaccination, respectively.

Bactericidal antibodies. Table 2 shows the bactericidal antibody titers against *N. meningitidis* strain N.150/88 (B:4:P1.15) in serum samples obtained before and after vaccination. Some of the serum samples were also tested against two additional B:4:P1.15 strains (N.44/89 and N.131/88), with comparable titers. For each age group, the increase in antibody titers after vaccination was statistically significant ($P < 0.05$). Also, the percentages of children with bactericidal titers of >1:4 pre- and postvaccination are shown. The postimmunization antibody levels in children less than 24 months of age were significantly lower than those of older children ($P < 0.05$). No differences in bactericidal titers or fold increases in children

TABLE 3. Geometric mean bactericidal titers against *N. meningitidis* N.150/88 (B:4:P1.15) in individuals with and without detectable prevaccination bactericidal activity

Age group (mo)	Log 2 bactericidal titer[a]					
	Without activity			With activity		
	Prevaccination[b]	Postvaccination	% ≥1:4[c]	Prevaccination[b]	Postvaccination	% ≥1:4[c]
3–23	0 (76)	1.7	19.7	1.3 (46)	2.5	26.1
24–47	0 (24)	4.3	54.0	2.5 (20)	6.1	45.0
48–83	0 (23)	4.6	43.5	4.6 (21)	19.7	67.0
Total	0 (123)	3.2	39.1	2.5 (29)	6.5	46.0

[a] A value of 1 was assigned to each titer of <1:2; thus, log 2 of 1 = 0.
[b] Values in parentheses are numbers of individuals.
[c] See Table 2, footnote *c*.

less than 1 year old compared with children 1 to 2 years of age could be seen (data not shown).

The influence of preimmunization antibody levels on bactericidal activity is shown in Table 3. For all age groups, except 24 to 47 months, there were significantly higher bactericidal titers after vaccination for children with demonstrable antibodies before vaccination than for those without such antibodies ($P < 0.05$). No significant differences in the percentages of children showing at least a fourfold increase in antibody titer among the three age groups were found when those with and without previous bactericidal antibodies were compared.

An analysis of the correlation between ELISA and bactericidal assay results is shown in Fig. 2. Poor correlation between the fold increases in antibody levels measured by these two assay techniques was evident. The correlation coefficient (r) varied from 0.2 to 0.4 among the three age groups.

Immunoblot studies. Figure 1 shows the binding profiles of IgG antibodies reactive with different OMPs in the serum samples of 13 vaccinees with bactericidal titers greater than 1:4. The antibodies bound most intensely to class 1, 4, and 5 OMPs. Postvaccination serum samples from individuals 30, 160, 370, 381, 415, 545, and 1069 showed distinct IgG binding to class 1 protein. High-molecular-weight IRPs were recognized in postvaccination serum samples from individuals 30 and 545. Two postvaccination serum samples with bactericidal titers of 1:256 and 1:128 showed no reactions with OMPs (data not shown).

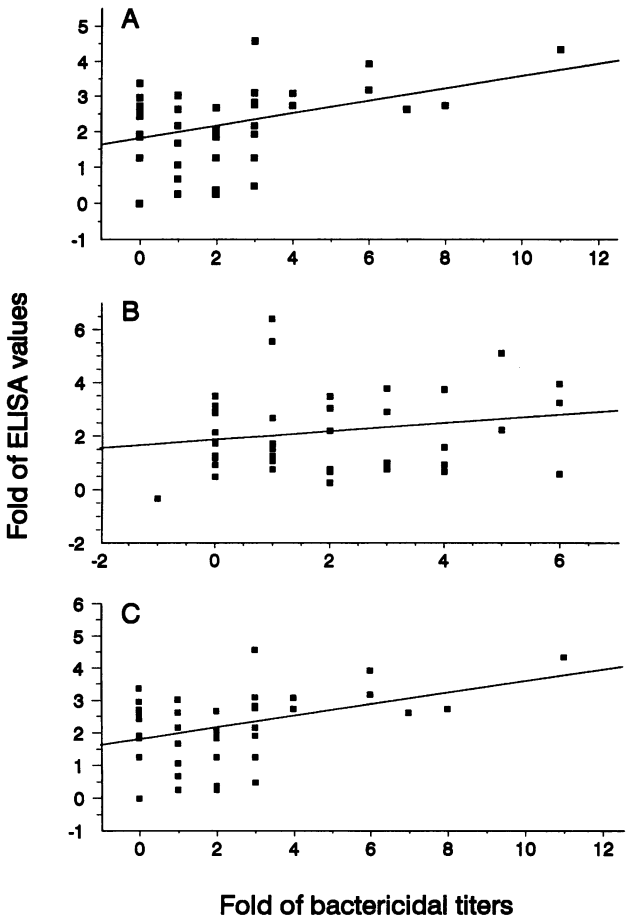

FIG. 2. Correlations between the fold increases of ELISA IgG antibody levels and bactericidal titers against *N. meningitidis* N.150/88 (B:4:P1.15) for children less than 24 months old ($r = 0.20$) (A), from 24 to 47 months old ($r = 0.20$) (B), and from 48 to 83 months old ($r = 0.41$) (C). Least-square regression lines are shown.

DISCUSSION

Bactericidal antibody against meningococcal group C polysaccharide has been shown to correlate with protection against meningococcal disease (10, 16). Some OMPs from group B meningococci induce bactericidal antibodies and have been used as alternative vaccines (4, 11). Several immunologic tests have been used in the evaluation of meningococcal group B protective immunity. These include ELISA studies, serum bactericidal and opsonic assays, and immunoblot studies (18, 19, 28, 35). The vaccination campaign of 2.4 million children from 3 months through 6 years of age in greater São Paulo provided an important and useful opportunity to study the correlation between immunogenicity and vaccine efficacy as estimated by the case-control study.

Our results showed that the functional immune responses of vaccinated children against group B meningococci are age dependent. There were significantly lower bactericidal titers for children less than 24 months of age and also a significantly lower percentage of these children with bactericidal antibodies (titer, ≥1:4) after vaccination compared with the percentages of older children. The percentage of children under 24 months of age with postvaccination titers of greater than 1:4 was only 13% compared with 47.7% for those over 24 months of age. It is interesting to note that the postvaccination seroconversion rate of children under 24 months of age (13%) was less than the naturally acquired levels of those over 24 months of age (Table 2). The age-specific incidence of group B meningococcal disease is much higher in children under 2 years of age, with a peak incidence in children under 1 year old. Our results are similar to those described by Frasch et al. (11), who studied the antibody responses of children to serotype 2a and 2b OMP vaccines combined with group B meningococcal polysaccharide but without adjuvant.

In agreement with our bactericidal assay results, the São Paulo case-control study showed that the estimated vaccine efficacy varied by age. The efficacy was 74% (95% confidence interval [95% CI], 16 to 92%) for children over 4 years old and 47% (95% CI, −72 to 48%) for those 24 to 47 months of age. The vaccine showed no protection for children less than 24 months of age (−37% and a 95% CI of −100 to 73%), and only 13% of the children showed postvaccination bactericidal titers of greater than 1:4 (24).

Greater increases in bactericidal titers after vaccination

were seen for children with preexisting antibodies than for those without such antibodies. Prior exposure to *Neisseria* proteins may explain the more vigorous response to vaccination. It is known that vaccination with a group B meningococcal outer membrane vaccine primes for a later anamnestic response (32). Although the immune responses in subjects without measurable preexisting antibodies were significantly lower than in those with demonstrable antibodies preimmunization, we found similar seroconversion rates as indicated by a minimum of fourfold increases in antibody titers after vaccination. The increase in antibody levels before vaccination was age dependent ($P < 0.05$). These observations support the role of naturally acquired immunity in protection against meningococcal disease.

On the basis of the estimated antibody levels to noncapsular surface antigens as measured by ELISA, the vaccine induced significant immune responses against group B *N. meningitidis* in vaccinees of all age groups. There were, however, some variations in response against the three B:4:P1.15 strains studied. The increase in antibody levels to OMV from strain N.150/88 after vaccination for children less than 24 months of age was significantly smaller ($P < 0.05$) than those for the other two age groups. The OMV from this strain showed smaller amounts of class 4 and 5 proteins in relation to class 1 protein. Perhaps there are important antigenic differences between strains of the same serotype that are isolated in different regions of the country.

The ELISA results with different group B meningococcal serotypes demonstrated more-intense antibody reactivity with 4:nt than with NT:P1.15 OMV. However, no differences in reactivity against these antigens could be seen by immunoblot studies (data not shown). This is not surprising because conformational differences and associations among major OMPs may account for the differences between ELISA and immunoblot reactivities.

The poor correlation between the overall antibody levels to various surface antigens estimated by ELISA and the bactericidal titers can be explained by the fact that the bactericidal assay measures only the subset of ELISA antibodies that are functionally important. Among important factors for bactericidal activity are the isotype and affinity of antibody molecules, as well as the ability of antibody molecules to activate complement (28, 31). Further studies are required to determine the predominant class of immunoglobulins induced by vaccination since they may have different functional activities.

The immunoblot studies showed considerable individual heterogeneity in antibody responses to vaccination. Considering that the primary antigenic epitopes detected by immunoblot are linear epitopes, class 1 and class 4 OMPs appeared to be the most reactive. An important finding was the correlation of IgG antibody binding to class 1 OMP in serum samples with high bactericidal activities (Fig. 1). This was observed for 7 of the 15 serum samples with titers of greater than 1:4. In this regard, others have correlated the antibodies induced to class 1 protein with protection (20, 30). The ability of antibodies to class 4 OMP to block otherwise bactericidal antibodies to specific cell surface antigens has not been well investigated (25). In general, pre- and postimmunization serum samples tested in our study showed weak reactivities with this protein. Serum samples from individuals 140 and 204, whose bactericidal titers postvaccination were 1:16 and 1:8, respectively, were reactive with class 4 OMP. The preimmunization serum sample from individual 204 clearly showed binding to this protein but did not have bactericidal activity. Weak IgG reactivities of 10 serum sample pairs with bactericidal titers of <1:4 were seen on immunoblots (data not shown).

Although the Cuban BC vaccine does not contain increased amounts of IRPs, one of 30 individuals (number 30) showed an increase in IgG binding to IRPs after vaccination. Also, the pre- and postimmunization serum samples from individual 545 showed reactivity with a high-molecular-weight protein. The human immune response to infection indicates that IRPs are expressed and immunogenic in vivo (4). There is considerable interest in exploring the use of one or more of these IRPs as vaccines (2, 9, 13).

These results demonstrate that the bactericidal assay is a good functional assay for the study of group B meningococcal vaccine immunogenicity, which appears to correlate with vaccine-induced protection. It is, nevertheless, also important to include the role of phagocytic killing in protection against meningococcal disease in future studies.

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