Vaccine-Induced Opsonophagocytic Immunity to Neisseria meningitidis Group B

J. S. Pleston and D. M. Granoff*

Center for Immunobiology and Vaccine Development, Children’s Hospital Oakland Research Institute, Oakland, California 94609

Received 29 January 2008/Returned for modification 21 February 2008/Accepted 12 March 2008

The role of opsonophagocytosis (OP) in protection against meningococcal disease is controversial because patients with deficiencies in terminal complement proteins whose sera support OP but not bactericidal activity (BA) are at greatly increased risk of disease. We assayed complement-mediated BA and OP bactericidal activity in sera from 32 adults immunized with an outer membrane vesicle vaccine given alone or combined with an investigational recombinant protein, genome-derived neisserial antigen (GNA2132). The sera were heat inactivated to remove internal complement activity, and BA was measured with exogenous nonimmune human serum as a complement source. OP was measured with human polymorphonuclear cells (PMNs) and C6-depleted complement, which without PMNs did not support BA. Before immunization, 9 to 19% of sera from subjects in both vaccine groups combined had BA titers of ≥1:4, which increased to 41 to 72% after immunization ($P < 0.01$ against each of three test strains). The percentages of sera with OP titers of ≥1:5 were 3 to 16%, which increased to 55 to 72% ($P < 0.001$ for each strain). Most postimmunization BA-positive sera were OP positive, but 10 to 37% of BA-negative sera also were OP positive. Comparing the two vaccine groups, there were no significant differences in the percentages of sera with BA or OP activity except for a higher percentage of OP against one strain in postimmunization sera from subjects in the combination vaccine group ($P = 0.02$). The data support independent roles for serum BA and OP bactericidal activity in protection against group B disease.

Serum complement-mediated bactericidal activity (BA) was shown by studies performed by Goldschneider et al. in the 1960s to confer protection against developing meningococcal disease (17). Subsequent studies also provided ample additional support for the protective role of serum BA (2–4). However, the Goldschneider study also found that a negative serum BA titer of <1:4 did not necessarily predict susceptibility to disease, since there were persons with negative titers who became colonized with the epidemic strain and who did not develop disease. Nevertheless, despite additional data that negative serum BA titers may underestimate the extent of immunity to meningococcal disease in the population (29, 30), a positive BA titer remains the only widely accepted correlate of protection against meningococcal disease (5). The possible role of opsonophagocytosis (OP) in protecting persons with negative serum BA titers remains controversial, in part because patients with deficiencies in terminal complement pathway proteins, whose sera support OP but not BA, have a greatly increased risk of acquiring meningococcal disease (8, 9).

The purpose of the present study was to investigate the potential independent roles of serum BA and OP in conferring protection against group B meningococcal disease. To address this question, we developed an OP bactericidal assay that used normal human serum depleted of C6 as an exogenous complement source, which in the absence of polymorphonuclear leukocytes (PMNs) did not support serum BA. We used this assay to measure OP bactericidal activity of stored serum samples from adults who had been participants in a previous group B meningococcal vaccine trial. The respective sera also were assayed for BA using C6-sufficient complement, which in the absence of PMNs elicited bacteriolysis by formation of a membrane attack complex.

(The data were presented in part at the 15th International Pathogenic Neisseria Conference, September 2006, Cairns, Australia.)

MATERIALS AND METHODS

Serum samples. Stored serum samples were available from 32 subjects, aged 18 to 50 years, who had been immunized as part of a previous vaccine trial. The subjects were from groups that had been randomly assigned to receive an outer membrane vesicle (OMV) vaccine ($n = 17$), which had been prepared by the Norwegian Institute of Public Health, Oslo (16), or the OMV vaccine combined with a recombinant protein ($n = 15$) (21, 31). The study was performed under the direction of one of the authors (D. M. Granoff) and was conducted in the Pediatric Clinical Research Center at Children’s Hospital and Research Center, Oakland. The recombinant protein, genome-derived neisserial antigen (GNA2132) (21) (encoded by the gene from Neisseria meningitidis strain 2996), was prepared by Chiron Vaccines, Siena, Italy (currently Novartis Vaccines). The dose of the OMV vaccine was 50 μg per injection, and the dose of the combination vaccine was 25 μg of OMV and 25 μg of recombinant protein. Both vaccines were adsorbed with aluminum hydroxide (1.65 mg per injection). Subjects in both groups were given three doses of vaccine, with each dose separated by a 1-month interval.

The sera assayed in the present study were from all subjects who had received either the OMV vaccine alone or the combination vaccine and who had completed the three doses of vaccine according to the protocol. We assayed the samples that had been obtained immediately before immunization and at 1 month after the third dose. The guidelines of the Institutional Review Board of Children’s Hospital and Research Center at Oakland were strictly adhered to using an approved protocol, and all of the subjects provided informed, written consent.

* Corresponding author. Mailing address: 5700 Martin Luther King Jr. Way, Oakland, CA 94609. Phone: (510) 450-7640. Fax: (510) 450-7915. E-mail: dgranoff@chori.org.

† Published ahead of print on 19 March 2008.
Neisseria meningitidis strains. We used three group B strains to measure BA and OP activity. One was H44/76-SL (B:15:P1.7,16 and ST 32), which also was used to prepare the OMV vaccine. This strain is referred to throughout the paper as H44/76. The other test strains were NZ98/254 (B:4:P1.7-2.4 and ST 41/44 complex), which was representative of the isolates that caused a recent epidemic in New Zealand (7), and S3032 (B:19:7:P112,16), which was from a patient hospitalized in the United States (15). NZ98/284 expressed a PorA with a heterologous serosubtype to that of the OMV vaccine strain, while the PorA of S3032 was heterologous at variable region 1 and homologous at variable region 2 (27).

Serum bactericidal assay. Following overnight incubation at 37°C in 5% CO₂ on chocolate agar (Remel, Rancho Cordova, CA), individual colonies of bacteria were inoculated into Mueller-Hinton broth supplemented with 0.5% glucose. The bacteria were grown for approximately 2 h to early log phase, pelleted by centrifugation, and washed and resuspended in Dulbecco's phosphate-buffered saline (Cellglo, 21-030-CV; Mediatech Inc., Herndon, VA) containing 0.5% glucose, 9 mM CaCl₂, and 4.9 mM MgCl₂·6H₂O and 1% (wt/vol) bovine serum albumin (LifeBlood Medical, Freehold, NJ). Serum was measured using sterile 96-well round-bottom plates (Costar) and was performed as described elsewhere (30). The source of complement was serum from a healthy adult with normal total hemolytic complement activity and no detectable BA against the test strains. After 60 min of incubation of the reaction mixture at 37°C, 12 μl was obtained for quantitative cultures. The percentages of bacteria surviving in wells containing complement and different dilutions of the test sera were calculated by comparing the respective CFU at 60 min with that at time zero in negative control samples. Serum BA titers were assigned by interpolation of the dilution that gave 50% bacterial survival. Note that bacteria incubated with a 1:4 dilution of a negative control serum and complement typically showed a 150 to 250% increase in CFU/ml during the 60 min of incubation.

Opsonophagocytic activity. On the day of the assay, blood was collected from one of three donors, each of whom was heterozygous for FcyRIIA (R131/H131 polymorphism) based on PCR, performed as described by Carlson et al. (6). For isolation of PMNs, 30 ml of blood anticoagulated with EDTA (BD vacutainers, K2) was added to an equal volume of Polymorphoprep gradient (Greiner Bio-One, Germany) according to the manufacturer’s instructions and centrifuged at 500 × g for 30 min at room temperature. The PMNs were separated and, after washing in buffer (without added calcium and magnesium), the cells were resuspended in 1 ml of buffer. Viability and purity were confirmed by staining with trypan blue and Türk’s stain, respectively.

The serum used as the complement source for the OP assay was the same as that used in the serum BA assay except that for the OP assay the serum was depleted of C6 by adsorption on an anti-C6 column. In brief, 1.2 mg/ml of murine immunoglobulin G2b anti-human C6 monoclonal antibody (MAb) (32) (kind gift from Dr. Wladimir Wiegand, Novartis, New York, NY) was added to an equal volume of Polymorphoprep gradient (Greiner Bio-One, Germany) and streptavidin-alkaline phosphatase as the detecting agent (Vector Labs, Burlingame, CA) (Fig. 1A). There was also no detectable C6 in the depleted serum as measured by Western blotting using a murine anti-human C6 biotinylated MAb (Quidel A706, San Diego, CA) and streptavidin-alkaline phosphate as the detecting agent (Vector Labs, Burlingame, CA) (Fig. 1A). We used a 1:1 dilution of undiluted serum using a total hemolytic assay. (B and C) Anti-PorA P1.4 MAb activity against N. meningitidis strain NZ98/254. (B) Bactericidal activity of the MAb with 40% nonimmune human serum depleted of C6 (closed triangles with dashed line), with 20% nonimmune human serum depleted of C6 (open triangles with dashed line), with 20% C6-depleted complement plus 0.5 μg/ml of purified human C6 (open circles with solid line), or with 20% nonimmune human serum that had not been depleted of C6 (Xs with solid line). (C) OP bactericidal activity of the MAb, based on 20% C6-depleted human complement and 40 PMNs per CFU of bacteria (open triangles with solid line), with 20% C6-depleted human complement without PMNs (solid triangles, dashed line), or with PMNs, antibody, and bacteria without C6-depleted complement (X, showing data point at one anti-P1.4 concentration).

There was no detectable C6 in the depleted serum as measured by Western blotting using a murine anti-human C6 biotinylated MAb (Quidel A706, San Diego, CA) and streptavidin-alkaline phosphate as the detecting agent (Vector Labs, Burlingame, CA) (Fig. 1A). There was also no detectable C6 in the depleted serum as measured by a total hemolytic assay (CH50; <1 U/ml) as measured by a total hemolytic assay: 789–001; Diamedix, Miami, CA). The C6-depleted complement did not support BA of an anti-PorA MAb (Fig. 1B). The addition of 0.5 μg/ml purified human C6 (CompTech, TX) to the C6-depleted serum complement source restored the majority of the hemolytic complement activity (216 U/ml compared with 298 U/ml before C6 depletion) and supported complement-mediated BA of the murine MAb to PorA (Fig. 1B).

The OP bactericidal assay was performed using a similar procedure to that described above for the serum BA except that C6-depleted human serum was used as the complement source in the OP assay and purified human donor PMNs were added to the reaction mixture (40 PMNs per CFU of bacteria). Although unable to support complement-mediated anti-PorA antibody bactericidal (Fig. 1B), the C6-depleted complement source supported opsonophagocytic anti-PorA bactericidal activity in the presence of human PMNs (Fig. 1C). In contrast, when incubated with human PMNs and anti-PorA antibody in the absence of added C6-depleted complement, there was no decrease in CFU/ml. Similar opsonophagocytic bactericidal activity of anti-PorA MAbs in the presence of C6-depleted complement and human PMNs was observed against the two other N. meningitidis test strains used in this study, H44/76 and S3032 (data not shown).

For determination of OP activity, test sera were assayed in duplicate at a final dilution of 1:5. A serum was considered positive if the percent survival of bacteria after 60 min of incubation was ≤50% compared with the CFU/ml at time zero. Typically, bacteria incubated for 60 min with a negative control serum, complement, and PMNs showed a 150 to 250% increase in CFU/ml. Controls in each assay included dilutions of a strain-specific relevant antiporin MAb against P1.7, P1.12, or P1.4 (NIHSC codes 01/514, 04/122 and 02/148, respectively; obtained from the National Institute for Biological Standards and Control, Potters Bar, United Kingdom), and OP-negative, low positive (1:5 to 1:10), and medium positive (OP titer of 1:12 to 1:20) human serum. The titers of the low- and medium-positive control sera were determined by assaying assay twofold dilutions and extrapolating the dilutions for 50% bacterial survival. Sixteen test sera...
Serum bactericidal antibody responses. Before vaccination, the percentages of sera with BA titers of $\geq 1:4$ in both vaccine groups combined were 19% (6/32), 16% (5/31), and 9% (3/32) against strains H44/75, S3032, and NZ98/254, respectively. One month after a third dose of vaccine, the percentages increased to 72% (22/32) against strain H44/76, which was used to prepare the OMV vaccine, 68% (21/31) against strain S3032, which was heterologous at PorA VR1 but shared the PorA VR2 serosubtype in common with that of the vaccine strain, and 41% (13/32) against NZ98/254, which had heterologous subtypes at both VR1 and VR2. The corresponding percentages after vaccination were significantly higher than before vaccination for each of the three strains ($P < 0.01$ by Fisher’s exact test).

Based on comparison of the serum BA responses of the two vaccine groups (Fig. 3A), there were no significant differences in the respective percentages of subjects with serum titers of $\geq 1:4$ before ($P > 0.5$) or after vaccination against each of the three test strains ($P = 0.07$ for strain S3032; $P > 0.5$ for strains H44/76 and NZ98/254). There also were no significant differences in the respective reciprocal geometric mean postimmunization titers (14, 8, and 8 in the OMV-alone vaccine group compared with 16, 10, and 5 in the OMV/GNA2132 combination vaccine group). The relatively low magnitudes of these titers may have reflected the short interval between injections (1 month). The results of the analyses described above were not appreciably changed if the BA results of the one preimmunization serum and one postimmunization serum with indeterminate OP activity were included.

RESULTS

Opsonophagocytic bactericidal activity. When measured with C6-depleted complement and added PMNs, the percentages of preimmunization sera with OP bactericidal titers of $\geq 1:5$ in both vaccine groups combined were 9% (3/32), 3% (1/31), and 16% (5/32) against strains H44/76, S3032, and NZ98/254, respectively. These percentages increased after three doses of vaccine to 72% (23/32) against strain H44/76, 55% (17/31) against strain S3032, and 59% (19/32) against strain NZ98/254 ($P < 0.001$ for each strain). When the respective results were stratified by vaccine group (Fig. 3B), the percentage of subjects with serum OP bactericidal activity after immunization was greater in the OMV/GNA2132 combination vaccine group against each of the three test strains, but the respective differences were significant only against strain H44/76 (93% [14/15] in the combination vaccine group, compared with 53% [9/17] in the OMV-alone vaccine group; $P < 0.02$).

Vaccine-induced antibodies that lack serum bactericidal activity can support opsonophagocytic bactericidal activity and vice versa. For each of the three strains there were examples of sera that had positive BA titers of $\geq 1:4$ but that were negative in the respective percentages of subjects with serum titers of $\geq 1:4$ before ($P > 0.5$) or after vaccination against each of the three test strains ($P = 0.07$ for strain S3032; $P > 0.5$ for strains H44/76 and NZ98/254). There also were no significant differences in the respective reciprocal geometric mean postimmunization titers (14, 8, and 8 in the OMV-alone vaccine group compared with 16, 10, and 5 in the OMV/GNA2132 combination vaccine group). The relatively low magnitudes of these titers may have reflected the short interval between injections (1 month). The results of the analyses described above were not appreciably changed if the BA results of the one preimmunization serum and one postimmunization serum with indeterminate OP activity against strain S3032 were included.
for OP bactericidal activity when tested with C6-depleted complement (Fig. 4B, strain S3032). There also were examples of sera with BA titers of <1:4 that were positive for OP bactericidal activity. Examples are shown in Fig. 4 for a subject given OMV vaccine (strain NZ98/254 [Fig. 4A]) and for a subject given the combination vaccine (strains H44/76 and NZ98/254 [Fig. 4B]). When the two vaccine groups were combined, the percentages of postimmunization BA-positive sera that were also positive for OP bactericidal activity were 87% (20/23), 76% (16/21), and 92% (12/13) for strains H44/76, S3032, and NZ98/254, respectively (P > 0.4) (Fig. 5). The corresponding percentages of BA-negative postimmunization sera that were positive for OP bactericidal activity were 33% (3/9), 10% (1/10), and 37% (7/19) (P > 0.3).

Table 1 summarizes the proportion of postimmunization sera with OP bactericidal activity when stratified by the presence or absence of a complement-mediated bactericidal titer of ≥1:4 and by vaccine group. Among subjects with serum BA titers of <1:4, there were trends for a higher proportion of the OMV/GNA2132 combination vaccine group to have OP bactericidal activity against each of the three test strains compared with the OMV-alone vaccine group, but the difference was significant only for strain H44/76 (P < 0.05).

**DISCUSSION**

A serum bactericidal titer of ≥1:4 measured with human complement is considered to be sufficient for protection against developing meningococcal disease (17; reviewed in reference 18). Although the magnitudes of the bactericidal titers in the present study were somewhat lower than previously reported, the percentages of subjects who developed serum BA titers of ≥1:4 against the different strains were similar to those previously reported in adults immunized with OMV vaccines (13, 14, 20, 25, 28). The addition of the recombinant GNA2132 to the OMV vaccine did not increase significantly serum BA responses, although there was a trend for a higher proportion of subjects in the combination vaccine group to have had protective serum BA titers against strain S3032 compared with the OMV-alone vaccine group (P < 0.07). The group given the combination vaccine also appeared to have greater OP bactericidal responses than the group given the OMV vaccine alone (for example, strain H44/76 [P < 0.02]), but the small sample sizes of each of the vaccine groups limited the statistical power to detect significant differences against the other two strains, if differences were present.

The GNA2132 gene encodes an *N. meningitidis* lipoprotein of unknown function that was identified by genomic studies (21). In mice, recombinant GNA2132 protein vaccine elicited serum antibody responses that were bactericidal with human complement against two of seven *N. meningitidis* strains tested (31). One of the susceptible strains was S3032, and one of the resistant strains was NZ98/254, both of which were used as test strains in the present study. Compared with the OMV vaccine alone group, the trends in the present study for increased serum bactericidal responses of subjects given the combination vaccine when measured against strain S3032, but not against strain NZ98/254, were consistent with the respective bactericidal responses of the GNA2132-immunized mice when measured against these strains. In the mouse study, anti-GNA2132 antibodies that lacked bactericidal activity passively protected infant rats against meningococcal bacteremia. Serum passive protective activity was not measured in the present study. However, among adults with negative serum bactericidal titers after vaccination, those given the combination OMV/GNA2132 vac-
cine may have had greater OP bactericidal activity than the group given the OMV vaccine alone (Table 1). This result would be consistent with the observed passive protective activity of mouse serum anti-GNA2132 antibodies against strains resistant to anti-GNA2132 serum bactericidal activity.

The primary purpose of the present study was to investigate the potential independent contributions of serum BA and OP bactericidal activity in conferring protection against group B meningococcal disease. Previous support for an independent protective role of OP came from studies of persons with late complement component deficiencies who were immunized with meningococcal polysaccharide vaccine and whose postimmunization sera supported OP bactericidal activity (1, 10, 23). Whether OP bactericidal activity alone could confer protection against group B strains, particularly if the antibodies were directed at noncapsular antigens (1, 8, 26), was unknown.

To investigate this question we performed an OP assay with C6-depleted complement, which in the absence of PMNs did not support bacteriolysis; the OP results therefore reflected bactericidal activity of PMNs that was independent of formation of a complement membrane attack complex and bacteriolysis. The lack of OP bactericidal activity in the great majority of the preimmunization sera was consistent with the high incidence of meningococcal disease in persons with terminal complement component deficiencies whose sera cannot support complement-mediated bactericidal activity (8, 11, 12, 22). The antigenic targets of naturally acquired bactericidal antibodies are likely to be diverse and different than those elicited by OMV vaccines, which elicited bactericidal antibodies primarily directed at PorA and Opc (25, 28). Our results indicated that the quality and/or concentrations of naturally acquired antibodies in most sera obtained before immunization were insufficient to activate complement-mediated bactericidal activity or OP bactericidal activity in the presence of C6-deficient complement.

The lack of naturally acquired serum OP bactericidal activity against group B strains when assayed with C6-depleted complement was opposite to that reported by Ross et al. in assays of OP activity of a C8-depleted human serum pool prepared from sera of 20 normal adults (26). Since the OP activity of the individual serum samples was not measured in that study, it is possible that the activity of the serum pool was disproportionately affected by a small number of individual sera with positive antibody titers. The lack of naturally acquired serum OP bactericidal activity with C6-depleted complement in the present study also was opposite to results we recently reported in unimmunized healthy adults using a whole blood bactericidal assay (30). In that study, 23 to 42% of the subjects with serum BA titers of <1:4 had whole blood OP bactericidal activity against group B strains. The whole blood assay combined both serum BA and OP bactericidal activity in the same assay, whereas the OP assay used in the present study was performed with C6-depleted serum as the complement source, which didn’t support complement-mediated BA. Also, both the test sera and the complement source were diluted 1:5 for the OP assay. Thus, the results reported in the present study likely underestimated the actual prevalence of OP bactericidal activity present in whole blood obtained before or after vaccination.

In contrast to the low prevalence of OP bactericidal activity in preimmunization sera, the majority of the postimmunization sera had serum BA and/or OP bactericidal activity when measured against the homologous strain used to prepare the OMV vaccine and against two heterologous group B strains. Thus, although naturally acquired antibodies in preimmunization sera infrequently elicited BA or OP, the higher antibody concentrations present in serum obtained after vaccination could support BA with C6-sufficient complement and/or OP bactericidal activity with C6-depleted complement. Although most postimmunization sera that were positive for complement-mediated BA activity also had OP activity against two of the test strains, H44/76 and NZ98/254, approximately one-third of postimmunization sera with BA titers of <1:4 had OP bactericidal activity (Fig. 5). These data suggest that OP bactericidal activity could have a role in protection against meningococcal disease that is independent of serum BA.

In previous studies, serum anticapsular antibodies elicited by meningococcal polysaccharide vaccination of patients with late complement component deficiencies also were reported to enhance OP bactericidal activity (1, 10, 23). That these OP antibodies can confer protection against disease also was suggested by a study reporting a lower relative risk of meningococcal disease in patients with terminal complement component deficiency who were immunized with meningococcal polysaccharide vaccine compared with the risk of disease in unvaccinated

### Table 1. OP bactericidal activity of postimmunization sera in relation to BA

<table>
<thead>
<tr>
<th>BA titer group and vaccine</th>
<th>Strain H44/76</th>
<th>Strain S3032</th>
<th>Strain NZ98/254</th>
</tr>
</thead>
<tbody>
<tr>
<td>BA titers of ≥1:4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OMV</td>
<td>9/12</td>
<td>75 (43–95)</td>
<td>7/9</td>
</tr>
<tr>
<td>OMV/GNA2132</td>
<td>11/11</td>
<td>100 (72–100)</td>
<td>10/13</td>
</tr>
<tr>
<td>BA titers of &lt;1:4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OMV</td>
<td>0/5</td>
<td>0a (0–53)</td>
<td>0/8</td>
</tr>
<tr>
<td>OMV/GNA2132</td>
<td>3/4</td>
<td>75b (19–99)</td>
<td>1/2</td>
</tr>
</tbody>
</table>

a The proportion of postimmunization sera with OP bactericidal activity, stratified by the presence or absence of a complement-mediated BA titer of ≥1:4 and by vaccine group. The CI was calculated from the binomial distribution.

b P < 0.05 by the Fisher exact test. Other differences in respective percentages of OP activity between the OMV and OMV/GNA2132 vaccine groups were not significant (P > 0.16).
ACKNOWLEDGMENTS

This study was supported by Public Health Service grant R01 AI46464 from the National Institute of Allergy and Infectious Diseases, NIH, and by a grant from Novartis Vaccines. The sera were obtained during a clinical trial performed in the Pediatric Clinical Research Center and supported by a grant from Novartis Vaccines and NIH grant M01-RR01271. The laboratory work was performed in a facility funded by Research Facilities Improvement Program grant number C06 RR-16226 from the National Center for Research Resources, NIH.

The serum samples were obtained as part of a vaccine trial conducted in the Pediatric Clinical Research Center of Children’s Hospital and Research Center at Oakland. We thank Bridget Canty and Betty Flores for enrolling the subjects and obtaining the serum samples during the clinical trial. Technical assistance for the assays was provided by Janet Wu, Ray Chen, and Tracy Wong.

REFERENCES