A Novel Mimetic Antigen Eliciting Protective Antibody to *Neisseria meningitidis*


*J Immunol* 2001; 167:6487-6496; doi: 10.4049/jimmunol.167.11.6487

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A Novel Mimetic Antigen Eliciting Protective Antibody to Neisseria meningitidis


Molecular mimetic Ags are of considerable interest as vaccine candidates. Yet there are few examples of mimetic Ags that elicit protective Ab against a pathogen, and the functional activity of anti-mimetic Abs has not been studied in detail. As part of the Neisseria meningitidis serogroup B genome sequencing project, a large number of novel proteins were identified. Herein, we provide evidence that genome-derived Ag 33 (GNA33), a lipoprotein with homology to Escherichia coli murein transglycosylase, elicits protective Ab to meningococci as a result of mimicking an epitope on loop 4 of porin A (PorA) in strains with serosubtype P1.2. Epitope mapping of a bacterial anti-GNA33 mAb using overlapping peptides shows that the mAb recognizes peptides from GNA33 and PorA that share a QTP sequence that is necessary but not sufficient for binding. By flow cytometry, mouse antisera prepared against rGNA33 and the anti-GNA33 mAb bind as well as an anti-PorA P1.2 mAb to the surface of eight of nine N. meningitidis serogroup B strains tested with the P1.2 serosubtype. Anti-GNA33 Abs also are bactericidal for most P1.2 strains and, for susceptible strains, the activity of an anti-GNA33 mAb is similar to that of an anticapsular mAb but less active than an anti-P1.2 mAb. Anti-GNA Abs also confer passive protection against bacteremia in infant rats challenged with P1.2 strains. Thus, GNA33 represents one of the most effective immunogenic mimetics yet described. These results demonstrate that molecular mimetics have potential as meningococcal vaccine candidates.

There is considerable interest in using molecular mimetic Ags to elicit protective immune responses to different pathogens (1–9). Mimetics also are being investigated for the treatment of cancer (10) and autoimmune diseases (11). For vaccines for prevention of infectious diseases, the mimetic approach has the greatest utility when the nominal Ag is toxic or difficult to purify, or when it is desirable to direct the immune response to a limited number of epitopes. Examples of experimental mimetic Ags include the lipoooligosaccharide of Neisseria gonorrhoeae (3), the fusion protein of respiratory syncytial virus (1), and the lipooligosaccharide of Neisseria meningitidis serogroup B (NmB)3 strains, and elicited serum bactericidal Ab responses (13). Among these was a lipoprotein, designated genome-derived Ag 33 (GNA33). The predicted amino acid sequence of GNA33 is homologous to that of membrane-bound lytic murein transglycosylase from Escherichia coli and Synechocystis sp. (14), and is highly conserved across N. meningitidis (13). Mice immunized with rGNA33 developed high-serum bactericidal Ab titers. The magnitude of the Ab response was similar to that of control animals immunized with an outer membrane vesicle (OMV) vaccine prepared from strain 2996. Because OMV vaccines are known to elicit protective serum Ab responses in humans (15–17), GNA33 was considered a promising candidate for development of a meningococcal vaccine capable of eliciting broad-based protection.

In this report, we show that GNA33 elicits protective Ab to NmB by mimicking a surface-exposed epitope on loop 4 of porin A (PorA) of strains with the P1.2 serosubtype. This unexpected identification of an unique mimetic Ag of PorA allowed us to compare the functional activity of Ab raised to a mimetic Ag to that raised by PorA, using in vitro and in vivo functional assays that historically have been relevant for predicting protection of meningococcal disease in humans (18).

Materials and Methods

Bacterial strains

The 22 N. meningitidis strains (21 in serogroup B and one in serogroup C) included in this study are summarized in Table I. The strains were isolated

Received for publication August 6, 2001. Accepted for publication September 25, 2001.

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0022-1767/01/$02.00

0222-1767/01/S02.00
from patients with meningococcal disease residing in different countries and were collected over a period of 36 years. Mutants of strains MC58, BZ232, and NMB (MC58ΔGNA33, BZ232ΔGNA33, and NMBΔGNA33, respectively) in which the gna33 gene was deleted and replaced by an antibiotic cassette were prepared by transforming the parent strain with the plasmid pBT3 Δerm. This plasmid contains the upstream and downstream flanking gene regions for allelic exchange and the ermC gene (erythromycin resistance). The absence of the gene in the genome of erythromycin-resistant colonies for each of the three strains was confirmed by Western blot analysis, performed as described below.

Expression and purification of GNA33

The gna33 open reading frame was amplified by PCR on chromosomal DNA from strain 2996 (22) with synthetic oligonucleotides used as primers. The amplified DNA fragment was cloned into pET-21b+ vector (Novagen, Madison, Wisconsin) to express the protein as His-tagged GNA33 or as a soluble protein without the signal and lipid modification sequences (gna33Δ). The expression of recombinant protein was evaluated by SDS-PAGE, performed as described below. The His-tagged fusion protein was purified by affinity chromatography on Ni²⁺–conjugated chelating fast-flow Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden) and the untagged form was purified by FPLC using a mono S ion-exchange resin (Amersham Pharmacia Biotech).

Preparation of polyclonal anti-GNA33 antiserum

To prepare antiserum against GNA33, 20 μg of purified His-tagged GNA33 or untagged rGNA33 was used to immunize 6-wk-old CD1 female mice (4–10 mice per group). The mice were obtained from Charles River Breeding Laboratories (Italy, Calco, Italy, or Hollister, CA). The recombinant protein was given i.p., together with CFA for the first dose and IFA for the second (day 21) and third (day 35) booster doses. Blood samples were taken on days 34 and 49. As a negative control, mouse polyclonal antiserum was prepared against E. coli proteins from the strain used to express rGNA33.

Preparation of mAbs

Four- to six-week-old female CD1 mice were immunized as described above, except that the third dose was given without adjuvant. Three days later, mice were sacrificed and their spleen cells were fused with myeloma cells P3 × 63Ag8.653 at a ratio of five spleen cells to one myeloma cell. After a 2-wk incubation in hypoxanthine/aminopterin/thymidine selective medium, hybridoma supernatants were screened for Ab binding activity by ELISA performed on microtiter plates coated with the noncapsulated N. meningitidis strain, M7 (23), which had been inactivated by treatment with
0.025% paraformaldehyde. Hybridomas secreting GNA33-specific Ab were cloned twice by limiting dilution and then expanded and frozen for subsequent use in tissue culture, or for ascites production in BALB/c mice.

The subclasses of the mAbs were determined using a mouse mAb isotyping kit (Amersham Pharmacia Biotech). Among the selected mAbs, one IgG2a anti-GNA33 mAb, designated mAb 25, was used in all of the binding and functional studies described below. This mAb was purified from mouse ascites by Hi-Trap affinity columns (Amersham Pharmacia Biotech) and, after exhaustive dialysis in PBS buffer, the concentration of the purified mAb was determined using a modified Lowry method with BSA as a standard (Bio-Rad DC Protein Assay; Bio-Rad, Munich, Germany). Specificity of mAb 25 binding was determined by Western blot using membrane proteins prepared from NmB strains MC58, B2Z32, and NMB, and their respective GNA33 knockouts (MC58ΔGNA33, B2Z32ΔGNA33, and NMBΔGNA33; see Results).

Binding of antisera to the surface of live encapsulated meningococci

The ability of monoclonal or polyclonal antisera elicited by the recombinant gna33 protein to bind to the surface of live pathogenic strains of NmB was determined using a flow cytometric detection of indirect fluorescence assay, performed as described previously (24). In brief, bacterial cells were grown to mid-log phase in Mueller-Hinton broth, harvested by centrifugation, and resuspended in blocking buffer (PBS containing 1% (w/v) BSA and 0.2% (w/v) sodium azide) at a density of ~10^6 cells per milliliter. Dilutions of test or control antisera or mAbs were then added and allowed to bind to the cells, which were then frozen on ice for 2 h. Following two washes with blocking buffer, the cells were incubated with FITC-conjugated F(ab')2 goat anti-mouse IgG (H and L chains) (Jackson Immunoresearch Laboratories, West Grove, PA) and fixed with 0.25% formaldehyde in PBS buffer, and the bacterial cells were analyzed by flow cytometry.

Positive control Abs included meningococcal-specific serotyping or subtyping mAbs (MN2C3B, MN16C13F4; Rijksinstituut Voor Volksgezondheid en Milieu) and SEAM 12, an anti-polysaccharide mAb that is specific for encapsulated group B strains. The negative control consisted of a mouse IgG2a anti-GNA33 mAb, designated mAb 25, was used as the complement source (our unpublished data). In a few experiments, the subclasses of the mAbs were determined using a mouse mAb isotyping kit (Amersham Pharmacia Biotech) and diisopropylcarbodiimide/N-hydroxysuccinimide activation (30). After the final cycle, all the peptides were N-terminally acetylated with 2% acetic anhydride. At the end of the synthesis, the peptides were desalted with 0.25% trifluoroacetic acid/triisobutyrlsilanate/water/dichloromethane (50/30/25).

Peptide binding assay

Cellulose-bound peptides were soaked in ethanol to prevent hydrophobic interactions between the peptides. Nonspecific binding was blocked by incubating cellulose sheets overnight at 4°C with 10 ml of 1% casein in TBS (50 mM Tris-HCl, 137 mM NaCl, and 27 mM KCl (pH 7.0)), containing 0.05% Tween 20. The sheets were incubated for 2 h at 37°C with the anti-GNA33 mAb25 (6 µg/ml) or an anti-PorA 1.2 mAb, followed by a 1/3000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG (Bio-Rad). Sheets were developed with bromo-4-chloro-3-indolyl-phosphate (Sigma-Aldrich, Steinheim, Germany) and 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl-tetrazolium bromide (Sigma-Aldrich) substrates in buffer (100 mM Tris (pH 8.9), 100 mM NaCl, and 2 mM MgCl2). Quantitative evaluation of the signal was obtained using a Umax Speedy II 2200 optical scanner (Umax Technologies, Fremont, CA).

Results

Binding of anti-GNA33 Abs to the bacterial cell surface as determined by indirect fluorescence flow cytometry

Polyclonal Abs obtained from CD1 mice immunized with rGNA33 (encoded by the gene from strain 2996) were tested for their ability to bind to live bacterial cells of various NmB strains by a flow cytometry binding assay. Fig. 1A shows binding of polyclonal anti-GNA33 antisera to four representative NmB strains, the parent strain, 2996 (P1.5,2), and three other strains, M3735 (P1.5,2), M4207 (P1.5), and MC58 (P1.7,16). The anti-GNA33 polyclonal antisera reacted only with strains 2996 and M3735. The anticapsular positive control mAb bound to all four strains, whereas the negative control antisera prepared from animals immunized with E. coli proteins showed only background binding. Fig. 1B shows the results of similar experiments measuring binding of the anti-GNA33 mAb25 to the bacterial cell surface of three strains (M3735 (P1.5,2), M4207 (P1.5), and MC58 (P1.7,16)). The mAb bound only to strain M3735 (P1.5,2).

Table I summarizes the results of flow cytometry experiments measuring the ability of anti-GNA33 antisera or mAb 25 to bind to the surface of live bacteria from 22 genetically diverse encapsulated meningococcal strains (21 in serogroup B and one in serogroup C). The anti-GNA33 Ab bound only to strains with the P1.5,2 or P1.2 serosubtypes (nine of nine vs zero of 13 strains with other PorA serosubtypes; p < 0.001). One of the nine positive strains, M986, showed lower binding than the other eight strains (vide infra). There was no binding to three strains (M4207, 1000,
and BZ83) that express the P1.5 epitope (present on loop 1 of PorA; see Ref. 31) but not the P1.2 epitope (loop 4). Also, there was no binding to strain M136, which does not express PorA (i.e., P1\textsuperscript{−}). These data indicate that binding of anti-GNA33 Ab to the bacterial surface correlates with expression of the PorA serosubtype P1.2.

**Western blot of total membrane fractions prepared from different N. meningitidis group B strains**

The apparent association between binding of anti-GNA33 Ab to the bacterial surface and expression of the P1.2 serosubtype was investigated further by SDS-PAGE Western blot of total membrane preparations from representative strains. Results from four serogroup B strains, two that were negative for anti-GNA33 surface binding by flow cytometry (NG3/88 (P1.7,1) and MC58 (P1.17,16)) and two that were positive (BZ232 and NMB (both P1.5,2)), are shown in Fig. 2. Data also are shown for total membrane preparations from three strains (MC58, BZ232, and NMB) in which the genes encoding GNA33 have been inactivated. In Fig. 2A, a single band with an apparent mass of ~48 kDa was detected by the anti-GNA33 mAb 25 in membrane preparations from the two non-P1.2 strains, NG3/88 (Fig. 2A, lane 3) and MC58 (Fig. 2A, lane 4). The band has an apparent molecular mass expected for rGNA33 (Fig. 2A, lane 1) and was absent in total protein prepared from the control E. coli strain (Fig. 2A, lane 2) or from the GNA33 knockout in strain MC58 (Fig. 2A, lane 5). In Fig. 2A, lanes 6 and 8 contain total membrane proteins prepared from strains BZ232 and NMB, respectively. Both of these strains have the PorA serosubtype P1.5.2. In each of the lanes there are two anti-GNA33-reactive bands. The higher 48-kDa band is absent from the GNA33 knockouts derived from BZ232 and NMB (Fig. 2A, lanes 7 and 9, respectively), a result confirming that this protein is GNA33.

**Fig. 2B** shows a Western blot of the same protein samples as described for Fig. 2A, but using the anti-PorA P1.2 mAb as the primary detecting Ab. As expected, there was no reactivity of the anti-PorA mAb with rGNA33 (Fig. 2B, lane 1), with the negative control E. coli proteins (Fig. 2B, lane 2), or with total membranes prepared from NmB strains that do not express PorA P1.2, such as BZ232 (Fig. 2B, lane 6), BZ232\textsuperscript{ΔGNA33} (Fig. 2B, lane 7), NMB (Fig. 2B, lane 8), and NMB\textsuperscript{ΔGNA33} (Fig. 2B, lane 9) that express...
PorA P1.2. The results suggest that the protein with an apparent mass of 41 kDa reacting with the anti-GNA33 mAb in Fig. 2A was PorA.

**Peptide mapping of the PorA and GNA33 epitopes recognized by the anti-GNA33 mAb 25**

To identify the specific amino acid sequence recognized by the anti-GNA33 mAb 25, we prepared overlapping linear decapeptides spanning the entire amino acid sequences of GNA33 and PorA loop 4 from strain 2996 (GenBank accession number X57180). The peptides that were positive (≥8 dye units) with mAb 25 are summarized in Table II. All eight of the positive GNA33 peptides share a tripeptide, QTP. The QTP sequence is also present in all five positive PorA P1.2 peptides that reacted with mAb 25. However, the QTP sequence is not sufficient for anti-GNA33 binding, as there was no mAb binding to three loop 4 peptides that contained QTP but lacked the preceding FVQ sequence.

To define the minimal peptide sequence from each protein that is sufficient for anti-GNA mAb 25 binding, we prepared progressively smaller GNA33 peptides beginning with AQAFQTPVHS (see Fig. 3A) and PorA P1.2 peptides beginning with TPAHFVQQTP (see Fig. 3B). The mAb bound strongly with GNA33 peptides containing FQTP and PorA P1.2 peptides containing FVQQTP, but not with any of the smaller peptides. The same minimal epitopes for each protein were identified by systematic alanine or glycine substitutions of amino acids contained within the relevant peptides of loop 4 of PorA and the GNA33 (data not shown).

These data suggest that the Abs elicited by rGNA33 have bactericidal activity against Nm strains expressing the P1.2 epitope as the result of cross-reactivity with the P1.2 epitope of PorA that contains the sequence QTP.

**Comparative binding of anti-GNA33 and anti-PorA P1.2 Abs to P1.2 Nmb strains**

The unexpected finding that anti-rGNA33 Abs cross-react with the PorA P1.2 epitope provided an opportunity to compare the activity of Ab raised to GNA33 with that elicited by PorA serosubtype P1.2. With one exception, the concentration-dependent binding of the anti-GNA33 mAb determined by flow cytometry was similar to that of a control anti-PorA P1.2 mAb for the nine P1.2 strains tested (see representative data for strains 8047 and B2232 in Fig. 4A). The exception, strain M986, showed relatively weaker anti-GNA33 Ab binding when compared with binding to the other P1.2 strains (see Fig. 4B). In contrast, binding by the anti-PorA P1.2 mAb was similar for all P1.2 strains, including M986.

The VR2 sequence type of strain M986 is reported to be P1.2 (GenBank accession number U92912), which is defined by a loop 4 sequence that includes the segment FVQQTPK, as opposed to VR2 type P1.2-2; see Table I). The VR2 type is based on the amino acid sequence of the particular P1.2 epitope (see Ref. 31 or http://mlst.zoo.ox.ac.uk for recently revised PorA VR designation conventions). Two other strains reported to have VR2 sequence type P1.2 (strains M3735 and M5682) showed strong anti-GNA33 Ab binding which, in each strain, was comparable to the respective binding of the control anti-PorA P1.2 mAb (see, for example, binding data with strain M3735 in Fig. 1A and strain M5682 in Fig. 4B). The VR2 sequence type of PorA loop 4 in strains M986, M3735, and M5682 was confirmed to be P1.2 by a repeated independent nucleotide sequencing experiment (M986 in our laboratory; M3735 and M5682 by C. Sacchi, personal communication). Therefore, the sequence difference (K→Q) does not appear to be sufficient to explain the decreased anti-GNA33 binding activity with strain M986.

**Comparative bactericidal activity of anti-GNA33 and anti-PorA P1.2 Abs against P1.2 Nm strains**

We compared the complement-dependent bactericidal activity of murine mAbs to PorA P1.2, rGNA33 (mAb 25), and serogroup B (SEAM 12) and C (mAb 181.1) polysaccharide capsules. With the exception of the serogroup C capsular mAb (subclass IgG3) that was used to test NmC strain M5954, the subclass of all of the other mAbs was IgG2a. The BC50 of the anti-PorA P1.2 mAb in the presence of human complement was ≥0.5 μg/ml for all nine strains. The corresponding BC50 values of the serogroup B anti-capsular mAb were higher, ranging from 5 to 12 μg/ml, and for the serogroup C mAb (strain M5954), <1 μg/ml.

As summarized in Table III, the bactericidal activity of the anti-GNA33 mAb was variable and was dependent on the complement source used. For three of the strains (8047, NMB, and M3735), BC50 values of the anti-GNA33 mAb in the presence of human complement ranged from 7 to 15 μg/ml. The respective values for these strains were similar to the bactericidal titers of the capsular Ab measured with this human complement source (serum from a healthy adult, without intrinsic bactericidal activity (25)).

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**Table II. Epitope mapping of anti-GNA33 mAb 25 against overlapping peptides prepared from GNA33 and loop 4 of PorA P1.2 (strain 2996)**

<table>
<thead>
<tr>
<th>GNA33a</th>
<th>Dye Units</th>
<th>Loop 4 of PorA P1.2a</th>
<th>Dye Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>QQDVSAQAFQ</td>
<td>0</td>
<td>YTPAHFVQQTP</td>
<td>0</td>
</tr>
<tr>
<td>DVSAQAFQTP</td>
<td>23</td>
<td>TPAHFVQQTP</td>
<td>8</td>
</tr>
<tr>
<td>VSQAQAFQTPV</td>
<td>27</td>
<td>PAFHVFQSSQ</td>
<td>10</td>
</tr>
<tr>
<td>SAQAFQTPVHS</td>
<td>29</td>
<td>AHFVQQTPQQ</td>
<td>14</td>
</tr>
<tr>
<td>AQAFQTPVHS</td>
<td>30</td>
<td>HVQQTPQQSQ</td>
<td>15</td>
</tr>
<tr>
<td>QAOFQTPVHSF</td>
<td>30</td>
<td>VFQTPQQSQQ</td>
<td>9</td>
</tr>
<tr>
<td>AFQTPVHSFP</td>
<td>24</td>
<td>VQPQTPQSQT</td>
<td>4</td>
</tr>
<tr>
<td>POFQTPVHSFAP</td>
<td>22</td>
<td>QQPQTPQSTL</td>
<td>0</td>
</tr>
<tr>
<td>PTVPVHSFAP</td>
<td>19</td>
<td>QQPQTPQSTLL</td>
<td>2</td>
</tr>
<tr>
<td>TPVPVHSFAP</td>
<td>2</td>
<td>TQPQTPQSTP</td>
<td>2</td>
</tr>
</tbody>
</table>

a The peptide sequences were considered positive for binding to the anti-GNA33 mAb if the developed spots were ≥8 dye units.
For the remaining six strains (2996, BZ232, M5545, M5682, M5954, and M986) there was no killing observed with the anti-GNA33 mAb in the presence of this human complement source (BC50/H11022 g/ml; Table III), or with a second human complement source (serum from a patient with agammaglobulinemia; BC50/H11022 30 g/ml). When infant rabbit serum was used as the complement source, all but one of the six strains that were resistant to killing with human complement were susceptible to anti-GNA33-induced lysis with the rabbit complement. The BC50 values of the susceptible strains when tested with rabbit complement ranged from 1 g/ml to 8 g/ml (see Table III). The exception was strain M986, where no killing was observed with the anti-GNA33 mAb when tested with human or rabbit complement (BC50 values, 150 and 30 g/ml, respectively). Lack of bacteriolysis for this strain may be related to the lower surface binding of the mAb as measured by flow cytometry (see Fig. 4B).

We tested the bactericidal activity of the polyclonal anti-rGNA33 antiserum against five of the strains using human complement (MAS). The respective bactericidal titers corresponded to the results measured with anti-GNA33 mAb 25 (see Table III).

**Passive protection by anti-GNA33 Abs in infant rats**

We investigated the ability of mouse anti-GNA33 Ab to confer passive protection against NmB bacteremia in an infant rat model. The NmB strains used included a strain susceptible to anti-GNA33 bacteriolysis in the presence of human or rabbit complement 8047, a strain resistant to anti-GNA33 bacteriolysis with human complement but susceptible with rabbit complement (BZ232), and a strain resistant to anti-GNA33 bacteriolysis in the presence of human or rabbit complement (M986; see Table III). Bactericidal activity of the anti-GNA33 mAb against the latter two strains also was tested using adult rat serum as a complement source. BZ232 was susceptible (BC50 value < 1 g/ml), whereas strain M986 was resistant (BC50 > 30 g/ml). The results of the passive protection experiments with the different strains are summarized in Table IV.

Experiment one was performed with polyclonal anti-GNA33 antiserum that had been used in our initial flow experiments (Fig. 1A) and that had also been tested for bactericidal activity (Table III). A total of 100 µl of a 1/5 or 1/25 dilution of the polyclonal mouse anti-rGNA33 antiserum mixed with 5.8 x 10^3 CFU of strain 8047 and given i.p. completely protected rats against bacteremia measured 18 h after the challenge. In the same experiment, all animals treated with 100 µl of a 1/5 or 1/25 dilution of the anti-GNA33 antiserum and challenged with 6.5 x 10^3 CFU of strain M986, a strain resistant to anti-GNA33 bacteriolysis, developed bacteremia. Despite lack of bactericidal activity, the geometric mean CFU per milliliter of blood of the animals treated with the anti-GNA33 antiserum and challenged with strain M986 was 10- to 20-fold lower than that of control animals treated with a negative control antiserum prepared against *E. coli* proteins (p = 0.02). Similar results were obtained in experiment two with the anti-GNA33 mAb 25. All six rats pretreated with 20 µg of mAb 25 i.p. at time 0 and challenged 2 h later with 3.5 x 10^3 CFU of strain M986 had bacteria present in blood samples obtained 18 h after challenge.

**FIGURE 3.** Binding of anti-GNA33 mAb 25 to progressively smaller peptides corresponding to segments from GNA33 (A) and PorA P1.2 (strain 2996) (B). The respective peptides shown were identified from mapping studies with overlapping 10-mer peptides prepared from each protein and shown to contain an epitope recognized by mAb 25 (see Table II).
However, the geometric mean CFU per milliliter was <0.3% of that of control animals pretreated with an irrelevant mAb (p < 0.02). In the same experiment, 20 μg per rat of the anti-PorA P1.2 mAb was completely protective against strain M986, and 2 μg per rat was partially protective (only one of six treated animals developed bacteremia).

In experiments three and four, rats were challenged with strain BZ232 (resistant to anti-GNA33 bacteriolysis with human complement but susceptible with rabbit or rat complement). The protective activity of the anti-GNA33 mAb against this strain was similar to or higher than that of the control anticapsular Ab and only slightly less than that of the anti-PorA P1.2 mAb.

### Table III. Bactericidal activity of anti-GNA33 Abs against different Nm strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>VR2 Sequence Type</th>
<th>Polyclonal Antisera (BC&lt;sub&gt;50&lt;/sub&gt; (1/titer))&lt;sup&gt;a&lt;/sup&gt;</th>
<th>mAb 25 (BC&lt;sub&gt;50&lt;/sub&gt; (μg/ml))&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Human complement&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Rabbit complement</td>
</tr>
<tr>
<td>8047</td>
<td>P1.2-2</td>
<td>≥16</td>
<td>15</td>
</tr>
<tr>
<td>NMB</td>
<td>P1.2-2</td>
<td>≥16</td>
<td>9</td>
</tr>
<tr>
<td>M3735</td>
<td>P1.2</td>
<td>ND&lt;sup&gt;d&lt;/sup&gt;</td>
<td>7</td>
</tr>
<tr>
<td>2996</td>
<td>P1.2-2</td>
<td>&lt;4</td>
<td>&gt;60&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
<td>BZ232</td>
<td>P1.2-2</td>
<td>&lt;4</td>
<td>&gt;150&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>M5682</td>
<td>P1.2</td>
<td>ND</td>
<td>60&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>M5954</td>
<td>P1.2</td>
<td>ND</td>
<td>60&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>M5545</td>
<td>P1.2-2</td>
<td>ND</td>
<td>60&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>M986</td>
<td>P1.2</td>
<td>&lt;4</td>
<td>&gt;150&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> BC<sub>50</sub>, concentration of mAb, or reciprocal dilution of antiserum that when incubated for 60 min with bacterial cells and 20% complement yielded a 50% decrease in CFU per milliliter compared to that at time zero.

<sup>b</sup> The human complement was serum from a healthy adult with no detectable anticapsular antibody by ELISA and no detectable intrinsic bactericidal activity when tested at 20 or 40% (25). The BC<sub>50</sub> values of the anti-PorA P1.2 mAb with this human complement ranged from 0.25 to 0.5 μg/ml. The BC<sub>50</sub> of the serogroup B anticapsular mAb (SEAM 12) with human complement for the eight serogroup B strains ranged from 5 to 15 μg/ml. The BC<sub>50</sub> for the serogroup C anticapsular mAb (181.1) for strain M5954 with human complement was <1 μg/ml.

<sup>c</sup> ND, Not done.

<sup>d</sup> No detectable bactericidal activity (BC<sub>50</sub> > 30 μg/ml) when tested with a second source of human complement (20% serum from a patient with agammaglobulinemia).  

FIGURE 4. Binding of murine mAbs to live encapsulated NmB strains as determined by indirect fluorescence flow cytometry. The mAbs tested are described in Fig. 1B. A, Concentration-dependent binding of anti-GNA33 mAb 25 to strains 8047 (BC<sub>50</sub> = 15 μg/ml with human complement) and BZ232 (BC<sub>50</sub> > 150 μg/ml with human complement). Both strains were susceptible to bacteriolysis when tested with rabbit complement (see Table III). B, Concentration-dependent anti-GNA33 binding to strains M986 (PorA VR<sub>2</sub>, type P1.2) and M5682 (PorA VR<sub>2</sub>, type P1.2), as compared with strain 8047 (PorA VR<sub>2</sub>, type P1.2-2). M986 was resistant to anti-GNA33 bacteriolysis (human or rabbit), M5682 was susceptible (human only), and strain 8047 was susceptible (human or rabbit).
Table IV. Anti-GNA33 Ab passive protection in infant rats challenged with N. meningitidis serogroup B strains 8047, M986, or BZ232

<table>
<thead>
<tr>
<th>Expt</th>
<th>Strain (challenge CFU per rat)</th>
<th>Treatment*</th>
<th>Serum Dilution or Dose of mAb (µg) per Rat</th>
<th>No. positive/total</th>
<th>CFU/ml (geo. mean, 10^3)</th>
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<tr>
<td>1</td>
<td><strong>8047</strong>&lt;br&gt;(5.8 × 10^3)</td>
<td>Anticapsular mAb</td>
<td>2</td>
<td>0/5</td>
<td>&lt;0.001</td>
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<td></td>
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<td>Anti-GNA33 antiserum</td>
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<td>0/5</td>
<td>&lt;0.001</td>
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<td></td>
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<td>0/5</td>
<td>&lt;0.001</td>
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<td></td>
<td>Anti-E. coli antiserum</td>
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<td>5/5</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Irrelevant mAb</td>
<td>2</td>
<td>5/5</td>
<td>63</td>
</tr>
<tr>
<td>1</td>
<td><strong>M986</strong>&lt;br&gt;(6.5 × 10^3)</td>
<td>Anticapsular mAb</td>
<td>2</td>
<td>0/5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>5/5</td>
<td>19</td>
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<td></td>
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<td>5/5</td>
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<tr>
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<td><strong>M986</strong>&lt;br&gt;(3.5 × 10^3)</td>
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<td>1/6</td>
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<td>630</td>
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<td></td>
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<td>0/6</td>
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<td>6/6</td>
<td>&gt;500</td>
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<td>4</td>
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<td>8/8</td>
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* In experiment 1, bacteria were mixed together with antisera or control mAb immediately before the i.p. challenge. In experiments 2, 3, and 4, animals were treated i.p. with the mAb at time 0. Two hours later they were challenged i.p. with the bacteria. In both experiments, blood cultures were obtained 18 h after the challenge. The anticapsular control mAb was SEAM 3 (20).

** For calculation of geometric mean CFU per milliliter, animals with sterile cultures were assigned a value of 1 CFU/ml. In experiment 1, the geometric mean CFU per milliliter of the combined group of animals given a 1/5 or 1/25 dilution of anti-GNA33 antiserum and challenged with strain M986 (28.8 × 10^3) was lower than that of the combined group of controls given the irrelevant mAb or E. coli antiserum (350 × 10^3; p = 0.02). In experiments 2, 3, and 4, the geometric mean CFU per milliliter of the animals treated with the anti-GNA33 mAb was lower than that of controls given the irrelevant mAb (p < 0.02).

**Discussion**

We have shown that mouse Abs produced as the result of immunization with rGNA33 are able to mediate bacteriolysis of N. meningitidis strains in the presence of complement because of cross-reactivity of anti-GNA33 Abs with the P1.2 epitope of the porin protein, PorA. This result was unexpected because GNA33 and PorA have no significant sequence homology, are structurally and functionally unrelated, and are physically located in different bacterial substructures. Hence, GNA33 can be described as an immunologic mimic of PorA.

Molecular mimicry can be defined as the functional similarity of two unrelated molecules. With respect to Ab-Ag interactions, molecular mimetics may exhibit antigenic mimicry by binding to the same paratope as the nominal Ag. In some cases, antigenic mimetics exhibit immunogenic mimicry by eliciting a protective Ab response against a pathogenic organism (representative references include Refs. 1, 2, 4, 6, 9, 32, and 33). The majority of immunogenic mimetics reported have been either peptide or anti-idiotypic Abs for the treatment of cancer (reviewed in Ref. 10) or prevention of infectious diseases (selected examples include Refs. 1, 2, 4, 9, and 32). Although there are numerous publications in the field of mimetic vaccines for prevention of infectious diseases, few of the reports have been confirmed by independent laboratories. Also, there have been few studies comparing the binding and functional activity of Abs elicited by the mimetic with those of Abs raised to the nominal Ag. In this context, the molecular mimicry exhibited by GNA33 is exceptional. First, GNA33 is a non-Ig protein that, as described above, is unrelated to PorA. Second, rGNA33 elicits an Ab response that, in many respects, is similar in functional activity to that elicited by native PorA in OMV preparations. Third, the polyclonal mouse anti-rGNA33 antisera described in this work were prepared in two independent laboratories (Chiron, Siena, Italy, and Children’s Hospital Oakland Research Institute, Oakland, CA), and the bactericidal data from the polyclonal and monoclonal anti-GNA33 Abs were independently replicated in the two laboratories.

In previous studies, immunization with peptides corresponding to loop 4 of PorA P1.2 failed to elicit Abs that bound to the native protein or mediated bacteriolysis in the presence of complement (34). Presumably, the smaller peptide fragments were unable to adopt stable conformations present in the native porin. Similarly, immunization with rPorA expressed in E. coli or Bacillus subtilis failed to elicit bactericidal Ab unless the conformation of the surface-accessible PorA epitopes in the recombinant protein were reconstituted using liposomes or detergents (35, 36). These results suggest that the epitopes on PorA responsible for eliciting bactericidal Ab are conformational. In contrast, immunization with the rGNA33 mimetic elicited bactericidal Ab that cross-reacted with the P1.2 epitope of PorA loop 4. Unlike rPorA, this occurred when the rGNA33 protein used as the immunogen was simply mixed with Freund’s adjuvant, without the need for renaturation of the recombinant molecule.

Abs generated to rGNA33 bound to the surface of most serotype P1.2 Nmb strains as well as an anti-PorA mAb that defines the P1.2 epitope. For some strains, the bactericidal activity of the anti-mimetic Abs was similar to that of a mAb that binds to the
polysaccharide capsule. However, for reasons that are unknown, the anti-mimetic Abs were less active than the corresponding anti-P1.2 mAb in activating complement-mediated bacteriolysis in vitro. This difference was particularly striking when human complement was used in the assay. With human complement only one-third of the P1.2 strains tested were susceptible to anti-rGNA33 bacteriolysis, and these strains required on average 10- to 20-fold higher Ab concentrations for bacteriolysis than the corresponding anti-PorA P1.2 mAb (see Table III).

The clinical importance of the lack of bactericidal activity of anti-GNA33 Abs against some P1.2 strains when tested with human complement is unclear. With the exception of strain M986, all of the strains that were resistant to bacteriolysis when tested with human complement were highly susceptible when tested with rabbit (see Table III) or rat complement (data not shown). Anti-GNA33 Abs also were protective in infant rats challenged with different P1.2 strains (see Table IV). For some strains, such as B232, the relative protective activity observed with the anti-GNA33 mAb25 was similar to that observed with the anti-PorA P1.2 mAb. Furthermore, both polyclonal and monoclonal anti-GNA33 Abs conferred protection against strain M986, even though that strain was highly resistant to anti-GNA33 bacteriolysis in the presence of human, rabbit, or rat complement in vitro. In the absence of bacteriolysis, the most likely mechanism for the observed protection against strain M986 is opsonization. The lower functional activity of the anti-mimetic Ab with human complement, as compared with that of the anti-PorA Ab, may result from subtle differences in binding avidity, or to subtle differences in the surface location of the respective epitopes recognized by anti-GNA33 mAb 25 and the anti-PorA P1.2 mAb.

Considerable data indicate that immunization of humans with OMV vaccines elicits serum bacterial Ab responses and confers protection against developing meningococcal disease (reviewed in Ref. 37). In human infants immunized with OMV vaccines, the bactericidal Ab response is directed primarily against surface-exposed epitopes on PorA (38), primarily loops 1 and 4, which contain the most surface-exposed PorA epitopes on encapsulated meningococcal strains. In immunized adults, the protective Ab responses also appear to be directed against the Opc protein (39). The discovery that GNA33 exhibits immunologic mimicry of the PorA P1.2 epitope suggests that GNA33 may be a vaccine candidate for the prevention of disease caused by P1.2 strains, which represent ~8% of serogroup B isolates in the U.S. (40). Further, by substituting other PorA loops into GNA33 or into subdomains of GNA33, it may be possible to generate immunogenic mimetics of other serosubtype PorA epitopes that could be used as Ags in a multivalent meningococcal vaccine. Such a vaccine would have many advantages over vaccines based on using rPorA. For example, the preparation of such a rGNA33 vaccine would be greatly simplified as rGNA33 can be conveniently expressed in large amounts in noninfectious E. coli, without the need for detergent extractions, refolding, or reconstitution in lipid vesicles. Also, the epitope-containing segments of PorA variants from newly emerged NmB strains causing disease could be substituted into GNA33 as needed.

Acknowledgments

We thank Katherine Alter for performing flow cytometry assays and Claudio Sacchi (Centers for Disease Control, Atlanta, GA) for kindly resequencing the porA genes from several strains he provided for this study. We are grateful to Alexander Lucas for reviewing the manuscript and providing helpful suggestions.

References


