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A Novel Mimetic Antigen Eliciting Protective Antibody to *Neisseria meningitidis*[1]

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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 bactericidal 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. ***The Journal of Immunology,*** **2001, 167: 6487–6496.**

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 lipooligosaccharide of *Neisseria gonorrhoeae* (3), the fusion protein of respiratory syncytial virus (1), and conserved epitopes on different strains of HIV-1 (7) or hepatitis C virus (5).

A large number of studies have investigated the vaccine potential of mimetic Ags. For example, anti-idiotype vaccines are being tested in humans for the treatment of melanoma and colon cancer (reviewed in Ref. 10). In contrast, the experimental data, to date, on the use of mimetic vaccines for the prevention of infectious diseases have not been sufficiently compelling to justify testing in humans.

As part of the recently completed meningococcal serogroup B genome sequencing project (12), a large number of novel proteins were discovered that were conserved in sequence, appeared to be surface-exposed on encapsulated *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

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[3] Abbreviations used in this paper: NmB, *Neisseria meningitidis* serogroup B; PorA, porin A; GNA33, genome-derived Ag 33; OMV, outer membrane vesicle.

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Table I. *Binding of anti-GNA33 Abs to the surface of live, encapsulated* N. meningitidis *strains as measured by flow cytometry in relation to serological classification and PorA VR designation*

Nm strain	Country	Year	Serologic Classification[a]	PorA VR Designation (Sequence)[b]	Anti-GNA33[c]
M5954	United States	1997	C:2a:P1.2[e]	ND	+
M5682	United States	1999	B:2a:P1.5,2[e]	P1.5,2[e]	+
M986	United States	1963	B:2a:P1.5,2 (41)	P1.5,2 (41)	+
M3735	United States	1992	B:NT:P1.5,2[e]	P1.5-1,2[e]	+
M5545	United States	1998	B:NT:P1.5,2[e]	P1.5-4,2-2[e]	+
8047	United States	1978	B:2b:P1.5,2[d]	P1.5-2,2-2[d]	+
NMB	United States	1982	B:2b:P1.5,2 (23)	P1.5-2,2-2[d]	+
BZ232	The Netherlands	1964	B:NT:P1.2[f]	P1.5-2,2-2 (13)	+
2996	The Netherlands	1975	B:2b:P1.5,2 (41)	P1.5-1,2-2 (41)	+
M136[g]	United States	1968	B:16,11:P1− (41)	P1.5-1,2-2 (41)	−
M4207	United States	1997	B:10:P1.5[e]	P1.5-1,10-1[e]	−
1000	USSR	1989	B:NT:P1.5[d]	P1.5-1,10-4 (13)	−
BZ83	The Netherlands	1984	B:P1.5,10[f]	P1.5-1,10 (13)	−
NG6/88	Norway	1988	B:NT:P1.1[f]	P1.7-4,1 (13)	−
BZ198	The Netherlands	1986	B:NT:P.NST[f]	P1.7-4,4 (13)	−
S3446	United States	1972	B:19,14:P1.22,14 (41)	P1.22-1,14 (41)	−
IH5341	Finland	1985	B:15:P1.7,16 (42)	ND	−
CU385	Cuba	1980	B:4,7:P1.19,15 (41)	P1.19,15 (41)	−
SWZ107	Switzerland	1980	B:4:P.NST[f]	P1.22-1,14 (13)	−
H44/76	Norway	1976	B:15:P1.7,16 (41)	P1.7,16 (41)	−
NG3/88	Norway	1988	B:8:P1.7,1[f]	P1.7,1 (13)	−
MC58	United Kingdom	1985	B:15:P1.7,16 (43)	15:P1.7,16-2 (43)	−

[a] NST, non-serosubtypable with available mAbs; −, PorA expression not detectable by SDS-PAGE.
[b] Based on the revised PorA VR type designation nomenclature proposed by Sacchi et al. (31) and http://mlst.zoo.ox.ac.uk/Meningococcus.
[c] Measured with mouse polyclonal anti-GNA33 antisera and/or mAb 25.
[d] Typing based on ELISA assay performed at Children's Hospital Oakland Research Institute with NIBSC or RIVM mAbs. Loop 2 and 4 amino acid sequences were inferred from DNA sequencing in our laboratory (strains 8047, NMB, and M986).
[e] C. T. Sacchi, unpublished observation.
[f] M. Achtman, unpublished observation.
[g] Strain M136 contains the gene for PorA having the VR type P1.5-1,2-2 but does not express PorA protein and is, therefore, P1−.

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 allelic exchange with an antibiotic cassette were prepared by transforming the parent strain with the plasmid pBSUD33ERM. This plasmid contains the upstream and downstream flanking gene regions for allelic exchange and the *ermC* gene (erythromycin resistance). Briefly, the upstream flanking region (including the start codon) from −867 to +75 and the downstream flanking region (including the stop codon) from +1268 to +1744 were amplified from MC58 using the following primers: U33 forward, 5′-GCTCTAGAGATGAGTCGAA CACAATGAACAATGTCCTGA; U33 reverse, 5′-TCCCCCGGGCTCT TGCTTTGGCAGGCGGCGA-3′; D33 forward, 5′-TCCCCCGGGCAC GGGATATGTGTGGC-3′; D33 reverse, 5′-CCCGCTCGAGAGTAGG GACAACCGG-3′.

The fragments were cloned into pBluescript (Stratagene, Milan, Italy) and transformed into *E. coli* DH5α using standard techniques (19). Once all subcloning was complete, naturally competent NmB strains MC58, BZ232, and NMB were transformed by selecting a few colonies grown overnight on chocolate agar plates and mixing them with 20 μl of 10 mM Tris·HCl (pH 6.5) containing 1 μg of plasmid DNA. The mixture was spotted onto a chocolate agar plate, incubated for 6 h at 37°C, 5% CO_2, then diluted in PBS and spread on chocolate agar plates containing 7 μg/ml erythromycin. The absence of the *gna33* gene in the genome of erythromycin-resistant colonies for each of the three strains was confirmed by PCR using the primers (F33, GCTCTAGAGGGCGACGACAGGCGG-3′; and R33, 5′-CCCGCTCGAGTTACGGGCGGTATTCGG-3′) that correspond to the 5′-sense and 3′-antisense strands, respectively, of the *gna33* gene. Lack of GNA33 expression in the three strain isolates was confirmed by Western blot analysis, performed as described below.

mAb reagents

Abs used for flow cytometry, bactericidal, and in vivo protection experiments included the following: an anti-meningococcal PorA P1.2-specific subtyping mAb (MN16C13F4, subclass IgG2a) obtained from Rijksinstituut Voor Volksgezondheid en Mileu (Bilthoven, The Netherlands) or from W. Zollinger (Walter Reed Army Institute of Research, Washington, DC); and anti-polysaccharide mAbs specific for encapsulated serogroup B (20) (SEAM 12 (subclass IgG2a) and SEAM 3 (subclass 2b)) and serogroup C (mAb 181.1 (21), subclass IgG3). mAb 181.1 was provided by K. Stein (U.S. Food and Drug Administration, Bethesda, MD). The negative control consisted of a mouse IgG mAb (VIG10) of irrelevant specificity.

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 (rGNA33). 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 Ni^{2+}-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 antisera

To prepare antisera 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 (Italia, 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 × 63-Ag8.653 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, München, Germany). Specificity of mAb 25 binding was determined by Western blot using membrane proteins prepared from NmB strains MC58, BZ232, and NMB, and their respective GNA33 knockouts (MC58ΔGNA33, BZ232ΔGNA33, 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 $\sim 10^8$ cells per milliliter. Dilutions of test or control antisera or mAbs were then added and allowed to bind to the cells, which were maintained on ice for 2 h. Following two washes with blocking buffer, the cells were incubated with 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 Mileu) and SEAM 12, an anti-polysaccharide mAb that is specific for encapsulated group B strains. The negative control consisted of a mouse IgG mAb (VIG10) of irrelevant specificity, or mouse polyclonal antiserum prepared against *E. coli* proteins from the strain used to express rGNA33.

Complement-dependent bactericidal Ab activity

Bactericidal activity was measured as previously described (24). Except where noted, the complement source was human serum from a healthy adult (25) with no detectable anticapsular Ab to serogroup B or C polysaccharide as tested by ELISA, and no detectable intrinsic bactericidal activity against the target strains when tested at a final serum concentration of 20% or 40%. In the present studies, this human complement source together with different test sera gave similar bactericidal titers to those measured when human serum from a patient with agammaglobulinemia was used as the complement source (our unpublished data). In a few experiments described in *Results*, bactericidal activity also was measured using serum from a patient with untreated agammaglobulinemia (26), infant rabbit serum, or adult rat serum as complement sources.

Animal protection

The ability of anti-GNA33 Abs to confer passive protection against NmB bacteremia was tested in infant rats challenged i.p. The assay was performed as previously described (27). In each experiment, 5- to 8-day-old pups from litters of outbred Wistar rats (Charles River Breeding Laboratories, Raleigh, NC) were randomly redistributed to the nursing mothers. A total of four experiments were performed using three different NmB strains (M986, BZ232, and 8047). Each strain had been serially passaged three times in infant rats and stored frozen at −80°C in skim milk. On the day before challenge, freshly thawed bacteria were inoculated onto chocolate agar and grown overnight at 37°C in 5% CO_2. On the morning of the challenge, colonies were picked, inoculated into a broth culture, and grown and prepared as described above for the bactericidal assay. In experiment one, to enhance sensitivity of the assay, the bacteria from strain 8047 or M986 were suspended in different concentrations of anti-GNA33 or control antisera immediately before the challenge. A total of 100 μl of the suspensions was then administered i.p. Heparinized blood specimens were obtained by cardiac puncture 18 h after the bacterial challenge. Aliquots of 1, 10, and 100 μl of blood were plated onto chocolate agar. The CFU per milliliter of blood were determined after overnight incubation of the plates at 37°C in 5% CO_2. In experiments two, three, and four, animals were treated i.p. with anti-GNA33 mAb or control mAbs at time 0. Two hours later the animals were challenged i.p. with bacteria from strain M986 (experiment two) or BZ232 (experiments three and four). Blood samples were obtained 18 h later and quantitation of bacteremia was performed as described for experiment one.

SDS-PAGE and Western blots

Total cell extracts of meningococcal bacteria were prepared as follows. Single colonies were grown in 7 ml of Mueller-Hinton broth (Difco, Detroit, MI) supplemented with 0.25% glucose to an A_{620} nm of 0.5–0.7. The bacteria were collected by centrifugation at 5000 × *g* for 15 min and resuspended in PBS. After freeze-thawing, the bacterial suspension was mixed with sample buffer (0.06 M Tris-HCl (pH 6.8), 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) 2-ME) and boiled for 10 min. Purified proteins (0.5 μg/lane) or total cell extracts (25 μg) derived from meningococcal strains were loaded onto 12.5% SDS-polyacrylamide gels (28) and transferred to a nitrocellulose membrane (29). The bound proteins were detected with anti-GNA33 mAb 25 at a final concentration of 6 μg/ml, or a 1/100 dilution of an anti-PorA P1.2 mAb followed by a 1/2000 dilution of HRP-labeled anti-mouse Ig (DAKO, Glostrup, Denmark).

Peptide spot synthesis

Peptide spot synthesis was performed on an amino-polyethylene glycol-cellulose membranes (Abimed Analyes-Technik, Langenfeld, Germany) using a model ASP 222 automated spot synthesizer (Abimed Analyes-Technik) and diisopropylcarbodiimide/*N*-hydroxybenzotriazole activation (30). After the final cycle, all the peptides were N-terminally acetylated with 2% acetic anhydride. At the end of the synthesis the side chain protecting groups were removed using a mixture of trifluoroacetic acid/triisobutylsilane/water/dichloromethane (50/3/2/45).

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 2% 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 $MgCl_2$). 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. 1*A* shows binding of polyclonal anti-rGNA33 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 antiserum reacted only with strains 2996 and M3735. The anticapsular positive control mAb bound to all four strains, whereas the negative control antiserum prepared from animals immunized with *E. coli* proteins showed only background binding. Fig. 1*B* shows the results of similar experiments measuring binding of the anti-GNA33 mAb 25 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,

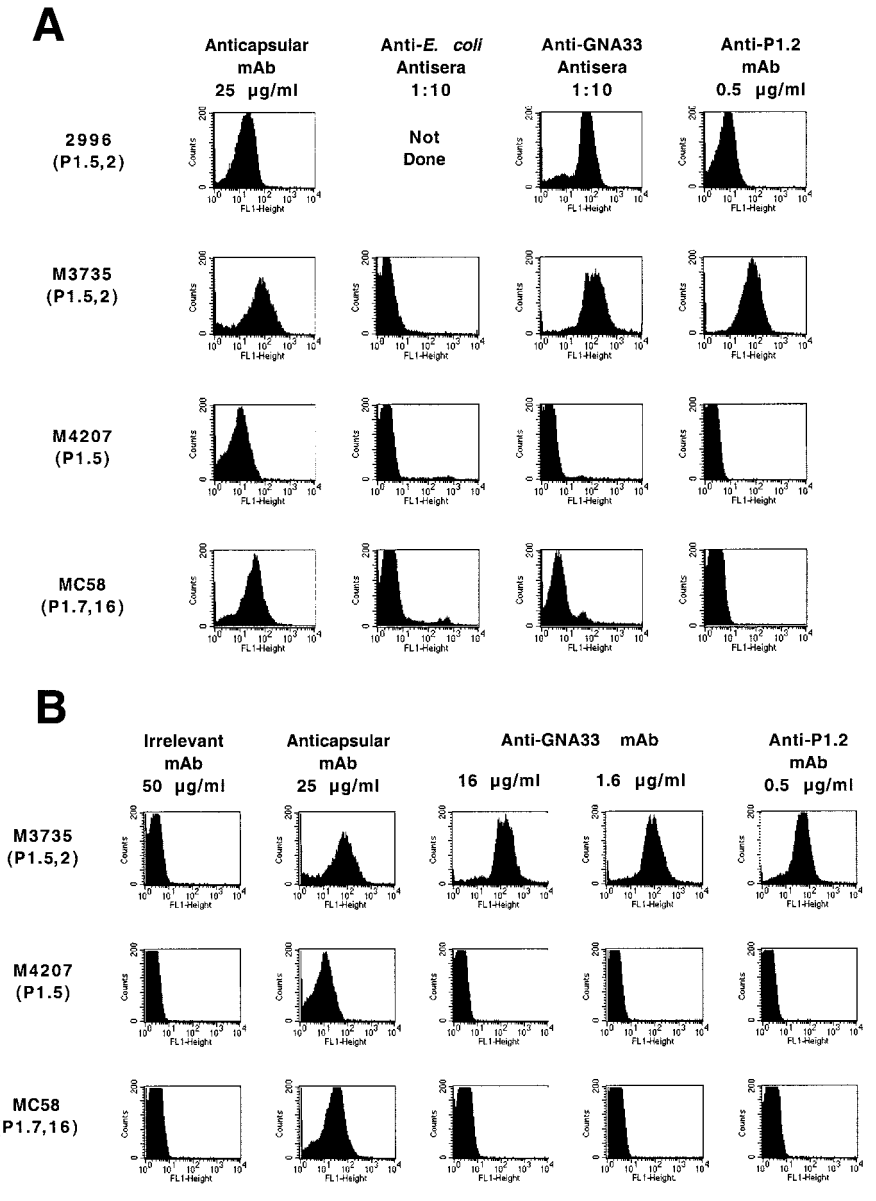

FIGURE 1. *A*, Binding of polyclonal anti-GNA33 antisera and control mAbs to live encapsulated NmB strains 2996, M3735, M4207, and MC58 as determined by indirect fluorescence flow cytometry. The control mAbs and antisera include an anti-serogroup B capsular-specific murine mAb (SEAM 12), an *N. meningitidis* serosubtype mAb anti-PorA P1.2, and polyclonal antisera from mice immunized with *E. coli* OMVs. *B*, Binding of anti-GNA33 mAb 25 and control mAbs to NmB strains M3735, M4207, and MC58. The murine control mAbs include a mAb having an irrelevant specificity (VIG10), and the same anticapsular and anti-PorA P1.2 mAbs described in *A*.

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−). 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 membranes prepared 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. 2*A*, 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. 2*A*, *lane 3*) and MC58 (Fig. 2*A*, *lane 4*). The band has an apparent molecular mass expected for rGNA33 (Fig. 2*A*, *lane 1*) and was absent in total protein prepared from the control *E. coli* strain (Fig. 2*A*, *lane 2*) or from the GNA33 knockout in strain MC58 (Fig. 2*A*, *lane 5*). In Fig. 2*A*, *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. 2*A*, *lanes 7* and *9*, respectively), a result confirming that this protein is GNA33.

Fig. 2*B* shows a Western blot of the same protein samples as described for Fig. 2*A*, 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. 2*B*, *lane 1*), with the negative control *E. coli* proteins (Fig. 2*B*, *lane 2*), or with total membranes prepared from NmB strains that do not express PorA P1.2 (Fig. 2*B*, *lanes 3–5*). However, a protein having an apparent mass expected for PorA was detected in total membrane preparations from strains BZ232 (Fig. 2*B*, *lane 6*), BZ232ΔGNA33 (Fig. 2*B*, *lane 7*), NMB (Fig. 2*B*, *lane 8*), and NMBΔGNA33 (Fig. 2*B*, *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. 2*A* 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. 3*A*) and PorA P1.2 peptides beginning with TPAHFVQQTP (see Fig. 3*B*). The mAb bound strongly with GNA33 peptides containing FQTPV 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 rGNA33 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 BZ232 in Fig. 4*A*). The exception, strain M986, showed relatively weaker anti-GNA33 Ab binding when compared with binding to the other P1.2 strains (see Fig. 4*B*). In contrast, binding by the anti-PorA P1.2 mAb was similar for all P1.2 strains, including M986.

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

GNA33[a]	Dye Units	Loop 4 of PorA P1.2[a]	Dye Units
QDVSAQAFQT	0	YTPAHFVQQT	0
DVSAQAFQTP	23	TPAHFVQQTP	8
VSAQAFQTPV	27	PAHFVQQTPQ	10
SAQAFQTPVH	29	AHFVQQTPQS	14
AQAFQTPVHS	30	HFVQQTPQSQ	15
QAFQTPVHSF	30	FVQQTPQSQP	9
AFQTPVHSFQ	24	VQQTPQSQPT	4
FQTPVHSFQA	22	QQTPQSQPTL	0
QTPVHSFQAK	19	QTPQSQPTLV	2
TPVHSFQAKQ	2	TPQSQPTVP	2

[a] The peptide sequences were considered positive for binding to the anti-GNA33 mAb if the developed spots were ≥8 dye units.

The VR_2 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 FVQQTPQ for strains 8047 and BZ232 (VR_2 type P1.2-2; see Table I). The VR_2 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 VR_2 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. 1*A* and strain M5682 in Fig. 4*B*). The VR_2 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 anticapsular mAb (subclass IgG3) that was used to test NmC strain M5954, the subclass of all of the other mAbs was IgG2a. The BC_{50} of the anti-PorA P1.2 mAb in the presence of human complement was ≤0.5 μg/ml for all nine strains. The corresponding BC_{50} values of the serogroup B anticapsular 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), BC_{50} 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 anticapsular Ab measured with this human complement source (serum from a healthy adult, without intrinsic bactericidal activity (25)).

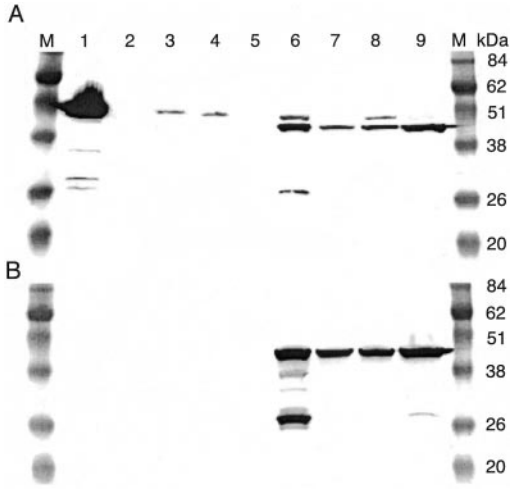

FIGURE 2. Western blots of rGNA33 and total proteins prepared from *E. coli* or *N. meningitidis* strains using anti-GNA33 mAb25 (*A*) or anti-PorA P1.2 (*B*) as the primary Ab. *Lane 1*, Recombinant GNA33; *lane 2*, total proteins prepared from *E. coli* strain used to express rGNA33; *lane 3*, NmB strains NG3/88; *lane 4*, MC58; *lane 5*, MC58ΔGNA33; *lane 6*, BZ232; *lane 7*, BZ232ΔGNA33; *lane 8*, NMB; and *lane 9*, NMBΔGNA33.

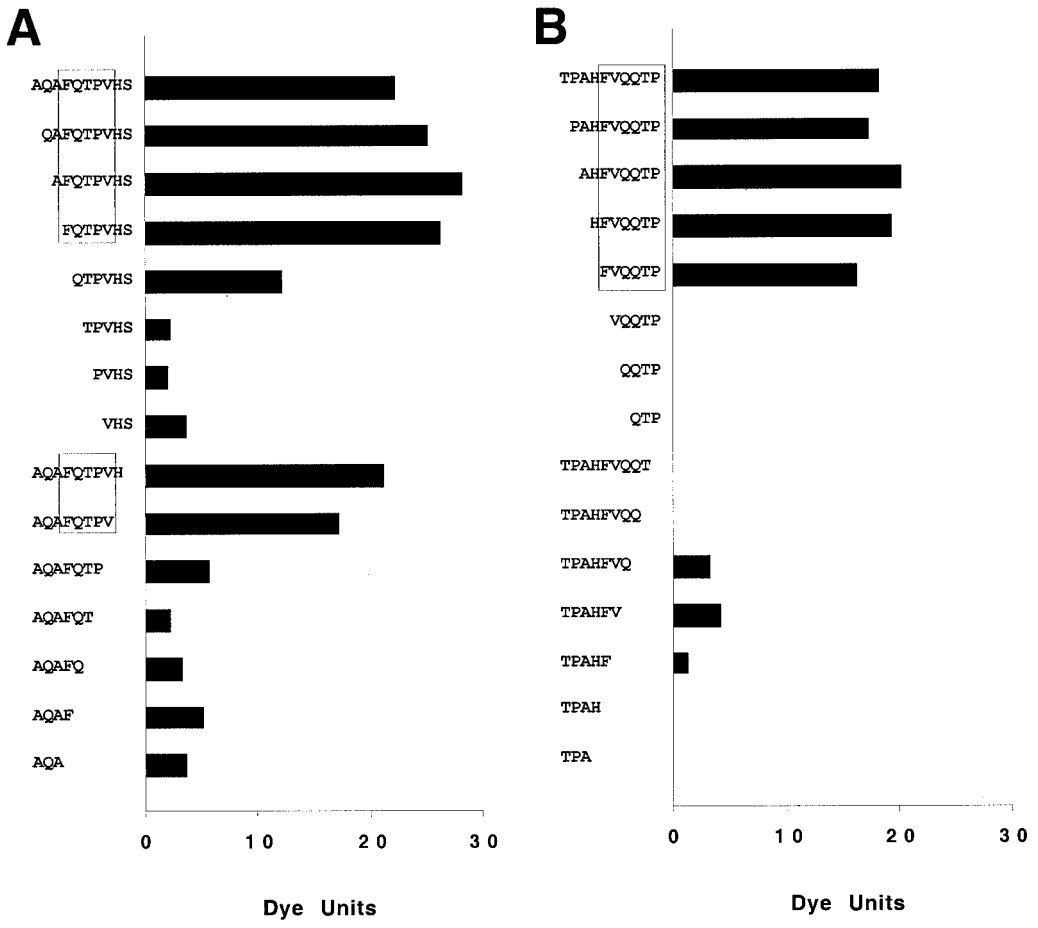

FIGURE 3. Binding of anti-GNA33 mAb 25 to progressively smaller peptides corresponding to segments from GNA33 (*A*) and PorA P1.2 (strain 2996) (*B*). The respective peptides shown were identified from mapping studies with overlapping 10-mer peptides prepared from each protein and shown to contain an epitope recognized by mAb 25 (see Table II).

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 ($BC_{50} > 60$ μg/ml; Table III), or with a second human complement source (serum from a patient with agammaglobulinemia; $BC_{50} > 30$ μg/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 BC_{50} values of the susceptible strains when tested with rabbit complement ranged from ≤ 1 μg/ml to 8 μg/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 (BC_{50} values, >150 and >30 μg/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. 4*B*). We also 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 (BC_{50} value < 1 μg/ml), whereas strain M986 was resistant ($BC_{50} > 30$ μg/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. 1*A*) 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 5.8×10^3 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 6.5×10^3 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 3.5×10^3 CFU of strain M986 had bacteria present in blood samples obtained 18 h after challenge.

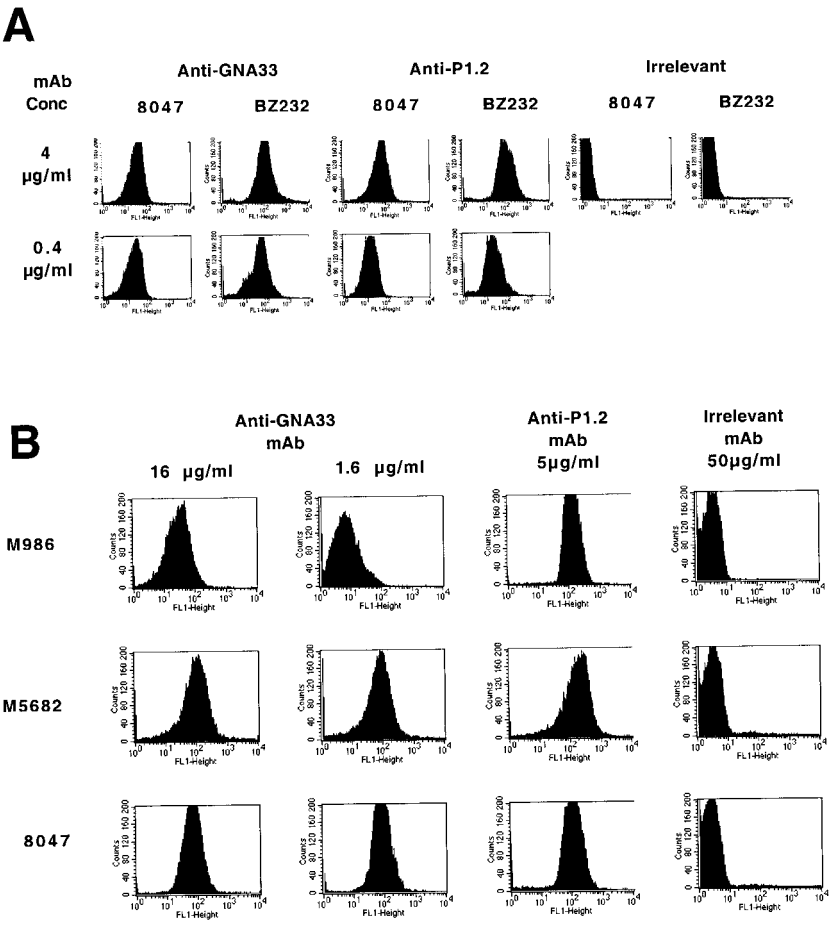

FIGURE 4. Binding of murine mAbs to live encapsulated NmB strains as determined by indirect fluorescence flow cytometry. The mAbs tested are described in Fig. 1*B*. *A*, Concentration-dependent binding of anti-GNA33 mAb 25 to strains 8047 (BC_{50} = 15 μg/ml with human complement) and BZ232 (BC_{50} > 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_2 type P1.2) and M5682 (PorA VR_2 type P1.2), as compared with strain 8047 (PorA VR_2 type P1.2-2). M986 was resistant to anti-GNA33 bacteriolysis (human or rabbit), M5682 was susceptible (rabbit only), and strain 8047 was susceptible (human or rabbit).

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*

Strain	VR2 Sequence Type	Polyclonal Antisera (BC_{50} (1/titer))[a]	mAb 25 (BC_{50} (μg/ml))[a]	
		Human complement[b]	Human complement[b]	Rabbit complement
8047	P1.2-2	≥16	15	<0.5
NMB	P1.2-2	≥16	9	ND
M3735	P1.2	ND[c]	7	ND
2996	P1.2-2	<4	>60[d]	<0.5
BZ232	P1.2-2	<4	>150[d]	<0.5
M5682	P1.2	ND	>60[d]	<0.5
M5954	P1.2	ND	>60[d]	1
M5545	P1.2-2	ND	>60[d]	8
M986	P1.2	<4	>150[d]	>30

[a] BC_{50}, 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.

[b] 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_{50} values of the anti-PorA P1.2 mAb with this human complement ranged from ≤0.25 to 0.5 μg/ml. The BC_{50} 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_{50} for the serogroup C anticapular mAb (181.1) for strain M5954 with human complement was <1 μg/ml.

[c] ND, Not done.

[d] No detectable bactericidal activity (BC_{50} > 30 μg/ml) when tested with a second source of human complement (20% serum from a patient with agammaglobulineumia).

Table IV. *Anti-GNA33 Ab passive protection in infant rats challenged with* N. meningitidis *serogroup B strains 8047, M986, or BZ232*

Expt.	Strain (challenge CFU per rat)	Treatment[a]	Serum Dilution or Dose of mAb (μg) per Rat	Blood Culture at 18 h: No. positive/ total	Blood Culture at 18 h: CFU/ml (geo. mean, 10^3)[b]
1	8047 (5.8×10^3)	Anticapsular mAb	2	0/5	<0.001
		Anti-GNA33 antiserum	1/5	0/5	<0.001
		Anti-GNA33 antiserum	1/25	0/5	<0.001
		Anti-*E. coli* antiserum	1/5	5/5	53
		Irrelevant mAb	2	5/5	63
1	M986 (6.5×10^3)	Anticapsular mAb	2	0/5	<0.001
		Anti-GNA33 antiserum	1/5	5/5	19
		Anti-GNA33 antiserum	1/25	5/5	41
		Anti-*E. coli* antiserum	1/5	5/5	408
		Irrelevant mAb	2	5/5	203
2	M986 (3.5×10^3)	Anticapsular mAb	20	1/6	0.002
		Anti-GNA33 mAb	20	6/6	1.873
		Anti-PorA P1.2 mAb	20	0/6	<0.001
		Anti-PorA P1.2 mAb	2	1/6	0.003
		Irrelevant mAb	20	6/6	630
3	BZ232 (7.1×10^3)	Anticapsular mAb	10	3/6	<0.056
		Anti-GNA33 mAb	15	0/6	<0.001
		Anti-GNA33 mAb	3	1/6	<0.006
		Anti-GNA33 mAb	0.6	5/6	0.282
		Anti-PorA P1.2 mAb	15	0/6	<0.001
		Anti-PorA P1.2 mAb	3	0/6	0.001
		Irrelevant mAb	15	6/6	>500
4	BZ232 (4.7×10^3)	Anti-GNA33 mAb	0.6	5/6	4.562
		Anti-PorA P1.2	3.0	0/6	<0.001
		Anti-PorA P1.2	0.6	0/6	<0.001
		Anti-PorA P1.2	0.12	3/7	0.022
		Irrelevant mAb	3	8/8	273

[a] 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).

[b] 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 antisera 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-idiotype 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 subtilus* 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 serosubtype 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 BZ232, 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 bactericidal 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 emergent NmB strains causing disease could be substituted into GNA33 as needed.

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