Antibody to Genome-Derived Neisserial Antigen 2132, a *Neisseria meningitidis* Candidate Vaccine, Confers Protection against Bacteremia in the Absence of Complement-Mediated Bactericidal Activity

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Genome-derived neisserial antigen 2132 (GNA2132) is a novel vaccine candidate that was identified during the *Neisseria meningitidis* group B strain MC58 genome-sequencing project. To assess the vaccine potential of GNA2132, we prepared antisera from mice immunized with recombinant GNA2132 (gene from strain NZ394/98). Anti-GNA2132 antibody bound to the surface of live bacteria from all 7 capsular group B or C strains tested and elicited deposition of human C3b on the bacterial surface. However, with human or infant-rat complement, anti-GNA2132 had no detectable bactericidal activity (titer, $1:4$) against the nominal strain, NZ394/98, and was bactericidal against only 2 of the other 6 strains tested. These differences between strains were unrelated to GNA2132 amino acid sequence or level of protein expression. Despite lack of bactericidal activity, anti-GNA2132 antiserum passively protected infant rats against meningococcal bacteremia after challenge with all 5 resistant strains. GNA2132 is thus a promising vaccine candidate for prevention of disease caused by *N. meningitidis*.

*Neisseria meningitidis* causes serious disease worldwide. In the United States and Europe, the organism remains the most common cause of bacterial meningitis in children and young adults [1, 2]. Strains with 5 different capsular groups—designated A, B, C, W135, and Y—cause most cases of serious invasive disease. Vaccines based on the capsular polysaccharide have been available for >30 years and currently offer protection against meningococcal disease caused by capsular group A, C, W135, and Y strains. At present, however, there is no broadly effective vaccine against disease caused by capsular group B strains [3, 4], which are responsible for the majority of cases of meningococcal disease in Europe [5] and approximately one-third of cases in the United States [6].

The group B capsular polysaccharide is structurally identical to a carbohydrate widely distributed in human tissues. Efforts to develop a group B capsular–based vaccine have been hampered by the risk of eliciting autoreactive antibody [7, 8]. Noncapsular antigens can also elicit protective antibody, but most vaccine candidates based on noncapsular antigens are limited by antigenic variability [3, 4].

Genome-derived neisserial antigen 2132 (GNA2132) is a putative surface lipoprotein that was identified by Pizza et al. during the group B strain MC58 genome-sequencing project [9]. The gene was detected in all 31 genetically diverse *N. meningitidis* strains tested and also was present in strains of *N. lactamica* and *M. hominis*. Serum antibody from mice immunized with recombinant GNA2132 (rGNA2132) elicited complement-medi-
ated bactericidal activity against group B strain 2996. The resulting titer, measured with rabbit complement, was of similar magnitude to that of control mice immunized with an outer-membrane vesicle vaccine that, in humans, is known to confer protective immunity against developing meningococcal disease. Thus, rGNA2132 is a promising vaccine candidate.

On the basis of gene sequences from 22 genetically diverse group B strains, there are segments of GNA2132 that are variable, whereas segments at the amino and carboxyl-terminal ends are highly conserved [9]. At present, it is unclear whether the conserved segments are sufficient to elicit cross-protective antibody against heterologous N. meningitidis strains. In the present study, we immunized mice with rGNA2132. The resulting antisera were tested for the ability to bind to the surface of live bacteria, to promote deposition of complement protein C3b/iC3b on the bacterial surface, and to elicit complement-mediated bactericidal activity. We also tested the antisera for the ability to confer passive protection against meningococcal bacteremia in infant rats challenged with different N. meningitidis strains.

**MATERIALS AND METHODS**

**Bacterial strains.** We investigated the activity of antirGNA2132 antisera against 7 N. meningitidis strains (table 1). These strains included strain NZ394/98, a representative isolate from an ongoing group B meningococcal epidemic in New Zealand [10], and the strain from which the gene encoding the recombinant protein used for the vaccine was cloned. Strain NZ394/98 was originally described as NZ98/254 (provided by D. Martin, Institute for Environmental Science and Research, Kenepura Science Center, Poirua, New Zealand). This strain and 4 other strains (2996, 8047, 4243, and 4335) were selected because they give reliable bacteremia in the infant-rat challenge model (see below). Two additional strains, S3032 and S3446, give inconsistent bacteremia in the rat model but are representative of strains susceptible to anti-GNA2132 bactericidal activity when tested with human complement (see below).

**DNA preparation and sequencing.** Colonies from an overnight growth of bacteria of each strain on chocolate agar were grown in 7 mL of Mueller-Hinton broth (Difco) supplemented with 0.25% glucose, to an absorbance at 620 nm (A_{600}) of 1.0. Genomic DNA was prepared by use of a commercial kit (Qiagen), according to the manufacturer’s directions. DNA used for sequencing was obtained by polymerase chain reaction (PCR) amplification of gna2132 in the genomic DNA preparation, by use of the following primers: for strains 4243 and 4335, forward 5'-CCGAATTCTGGGGGGCGGTTGG-3', reverse 5'-CTGTAAGCTTACCTGCTTTTTGCGC-3' (Operon); and, for strains S3032 and S3446, forward 5'-CTGAAATTCAGTGGACCCACTGTTAAG-3', reverse 5'-CTAAGCTTATCCTGCTTTTTTTGCGC-3' and forward 5'-CTGAAATTCAGTGGACCCACTGTTAAG-3', reverse 5'-CTAAGCTTACCTGCTTTTTGCGC-3'. Amplified DNA was purified by use of QIAquick PCR purification kit (Qiagen), according to the manufacturer’s directions. Sequencing was done with a BigDye Terminator cycle sequencing kit (Applied Biosystems) and an automated sequencer (model 3700, Applied Biosystems).

**Table 1. Summary of Neisseria meningitidis strains and anti–genome-derived neisserial antigen 2132 (GNA2132) bactericidal activity.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Country</th>
<th>Year(s)</th>
<th>Serologic classification</th>
<th>PorA VR designation (sequence)</th>
<th>Anti-GNA2132, 1/titer</th>
<th>BC_{50} IgG/mL</th>
<th>Murine MAb and human complement, BC_{50} µg/mL</th>
<th>Anti-GNA2132 IgG surface binding, by flow cytometry</th>
</tr>
</thead>
<tbody>
<tr>
<td>NZ394/98a</td>
<td>New Zealand</td>
<td>Late 1990</td>
<td>B:4;4</td>
<td>7–2,4</td>
<td>&lt;41</td>
<td>2000</td>
<td>100</td>
<td>0.04</td>
</tr>
<tr>
<td>2996</td>
<td>UK</td>
<td>1980s</td>
<td>B:2b;5,2</td>
<td>5,2</td>
<td>&lt;41</td>
<td>1800</td>
<td>80</td>
<td>0.24</td>
</tr>
<tr>
<td>8047</td>
<td>US</td>
<td>1978</td>
<td>B:2b;5,2</td>
<td>5–1,2–2</td>
<td>&lt;41</td>
<td>2000</td>
<td>50</td>
<td>0.06</td>
</tr>
<tr>
<td>4243</td>
<td>US</td>
<td>1995</td>
<td>C:2a;5,2 (OAc+)</td>
<td>ND</td>
<td>&lt;41</td>
<td>1500</td>
<td>0.10</td>
<td>0.70</td>
</tr>
<tr>
<td>4335</td>
<td>US</td>
<td>1995</td>
<td>C:2a;5,2 (OAc−)</td>
<td>ND</td>
<td>&lt;41</td>
<td>200</td>
<td>0.10</td>
<td>0.08</td>
</tr>
<tr>
<td>S3446</td>
<td>US</td>
<td>1972</td>
<td>B:19;14, 23, 14</td>
<td>23,14</td>
<td>329</td>
<td>80009</td>
<td>40</td>
<td>ND</td>
</tr>
<tr>
<td>S3032</td>
<td>US</td>
<td>1973</td>
<td>B:19;7,12,16</td>
<td>12,16</td>
<td>&gt;64</td>
<td>2500</td>
<td>150</td>
<td>ND</td>
</tr>
</tbody>
</table>

**NOTE.** BC_{50}, concentration of capsular monoclonal antibody (MAb) that killed 50% of bacterial cells after incubation with human complement; ND, not determined; VR, variable region.

a On the basis of the proposed PorA VR type designation nomenclature of Sacchi et al. [33].
b SEAM 12 [12] was used for group B strains; MAb C2/706 [13] was used for group C strains 4243 and 4335.
c Anti-Por A is anti-P1.2 or anti-P1.4.
d +, See figure 1B (NZ394/98) for example; ±, see figure 1B (2996) for example.
e Gene used to prepare recombinant GNA2132 protein.
f Titer of <1:4 also present when tested with infant-rat complement.
g Titer of <1:4 and <1:16, respectively, when measured with human or rabbit complement against strain S3446 in which the gene encoding GNA2132 was inactivated.
manufacturer’s directions. PCR products were ligated into either pBluescript II KS (±) phagemid (Stratagene) or pGEM(3zf−) (Promega), by use of the HindIII and EcoRI sites. After selection of individual transformants, plasmid DNA was isolated by use of a plasmid purification kit (Qiagen). Sequencing of DNA from both strands, from each clone, was performed by Davis Sequencing (Davis, CA) or Bionexus (Oakland, CA). Alternatively, the DNA sequence of gna2132 was obtained by direct PCR sequencing of genomic DNA, which was performed by the DNA-sequencing facility at Chiron Vaccines, Siena, Italy.

**GNA2132 vaccine.** The gna2132 open-reading frame (ORF) encoding aa 25–492 was amplified by PCR on chromosomal DNA from strain NZ394/98 by use of synthetic oligonucleotides as primers (forward, CGCGATTCGAAGCCGCATGTATTTGCG [Nhe site]; reverse, CCCGCTCGAGTCTCTGCTCCTTTTTGCC [Xho site]; restriction sites are underlined). The amplified DNA fragment was cloned into the vector pET-21b+ (Novagen) to express the protein as His-tagged (GNA2132-HT). *Escherichia coli* strain BL21 (DE3) harboring the plasmid pET-GNA2132-HT was grown at 37°C, to an A620 of 0.6–0.8, in Luria-Bertani medium containing ampicillin (100 μg/mL). Expression of recombinant protein was induced with 1 mmol/L isopropyl-β-D-thiogalactopyranoside (Sigma Chemical), and the culture was shaken for an additional 3 h at the same temperature. The expression of recombinant protein was evaluated by SDS-PAGE. After induction, cells were collected by centrifugation at 8000 × g for 15 min at 4°C, and then the pellet was resuspended in 50 mmol/L sodium phosphate buffer (pH 8) containing 300 mmol/L NaCl, 10 mmol/L imidazole, and a mixture of EDTA-free protease inhibitors (Roche). All subsequent procedures were performed at 4°C. Cells were disrupted by sonication. Debris and membranes were pelleted by centrifugation at 16,000 g for 30 min and then were discarded. GNA2132-HT was purified by affinity chromatography on Ni2+-conjugated chelating fast-flow Sepharose (Amersham-Pharmacia Biotech), by loading the supernatant on the metal-chelate affinity chromatography column. The column was extensively washed with 10 mmol/L followed by 20 mmol/L imidazole, in the same buffer used for the pellet resuspension. The GNA2132-HT protein was purified in a single-step elution with 250 mmol/L imidazole, in the same buffer.

**Polyclonal anti-rGNA2132 antisera.** Fifteen micrograms of recombinant GNA2132 protein was used to immunize groups of 4–6-week-old female CD-1 mice (6 mice/group). Injections were given intraperitoneally (ip). Complete Freund’s adjuvant (CFA) was used for the first dose, and incomplete Freund’s adjuvant (IFA) was used for 2 subsequent, booster doses given at 3-week intervals. Control mice were immunized with 3 injections of the respective adjuvant without vaccine. For assessment of serum antibody responses, individual mouse serum samples obtained 3 weeks after the third immunization were pooled.

**Binding of antisera to the surface of live encapsulated meningococci.** The ability of anti-rGNA2132 antisera to bind to the surface live *N. meningitidis* was determined by flow cytometric detection of indirect fluorescence assay, performed as described elsewhere [11]. Fluorescein isothiocyanate (FITC)–conjugated F(ab’)2 fragment goat anti–mouse IgG (H+L) (Jackson Immune Research) was the reagent used for fluorescence labeling. Positive control antibodies included SEAM 41 and C2/706, antipolsaccharide monoclonal antibodies (MAbs) specific for encapsulated group B and C strains, respectively. SEAM 41 was prepared in our laboratory [12], and MAb C2/706 [13] was provided by K. Stein (US Food and Drug Administration, Bethesda, MD). The negative control consisted of the pooled serum obtained from control mice immunized with adjuvant alone.

**Binding of human complement to the surface of live encapsulated meningococci.** Anti-GNA2132 antibody-dependent deposition of C3b or iC3b, on the bacterial surface of live *N. meningitidis* bacteria, was determined by flow cytometry. Bacterial cells were grown to mid-log phase in Mueller–Hinton broth supplemented with 0.25% glucose, were harvested by centrifugation, and were resuspended in veranol buffer (5 mmol/L barbitol, 145 mmol/L NaCl, 0.5 mmol/L MgCl2, and 0.15 mmol/L CaCl2 [pH 7.4]) containing 1% (wt/vol) bovine serum albumin (BSA), to a density of 107 cells/mL. To inactivate intrinsic complement, all antisera were heated for 30 min at 56°C. Dilutions of test or control mouse antisera (typically serial 4-fold dilutions of 1:100–1:1600 dilution) and 5% (vol/vol) complement were added to the cells, and cells were maintained for 30 min at room temperature. The complement source was human serum from a healthy adult with no detectable anticapsular antibody to group B or C polysaccharide when tested by ELISA, no detectable bactericidal activity, and no detectable deposition of complement on the bacterial surface of different strains, in the absence of added antibody. After 2 washes with veranol buffer, the cells were incubated with FITC-conjugated sheep anti–human C3c (BioDesign) for 20 min at 4°C. Anti-C3c reacts with both C3b and iC3b. The bacteria were harvested by centrifugation, were washed twice with veranol buffer, were fixed with 0.25% (vol/vol) formaldehyde in PBS buffer, and were analyzed by flow cytometry. Positive and negative controls were those described above for measurement of antibody binding by flow cytometry. As an additional negative control, each dilution of antiserum was tested for complement deposition in the presence of the human complement source that had been heat-inactivated by heating for 30 min at 56°C.

**Complement-dependent bactericidal antibody activity.** The method used for the bactericidal assay has been described in detail elsewhere [14]. In brief, the test organism was grown for ∼2 h at 37°C in Muller–Hinton broth supplemented with...
0.25% glucose, to an $A_{620}$ of $\sim 0.6$. After washing the bacteria twice in Gey’s buffer (pH 7.4) containing 1% (wt/vol) BSA, $\sim 300–400$ cfu were added to the reaction mixture. The final reaction mixture of 60 $\mu$L contained 20% (vol/vol) complement and serial 2-fold dilutions of antisera in Gey’s buffer. Serum bactericidal titers were defined as the serum dilution that resulted in a 50% decrease in colony-forming units per milliliter after incubation of bacteria in the reaction mixture for 1 h at 37°C, compared with that at time 0. Typically, bacteria incubated with the negative control antibody and complement showed a 150%–200% increase in colony-forming units per milliliter during the 1 h of incubation. The complement sources consisted of the normal human serum described above, pooled serum samples from 5–7-day-old out-bred Wistar rats (Charles River), or pooled serum from young rabbits (Cedarlane). None of these complement sources had detectable intrinsic bactericidal activity (i.e., 2-fold higher than the concentration used to measure bactericidal activity in the test antisera) when tested at either 20% or 40%.

**Passive protection in infant rats.** The ability of the anti-GNA2132 antiserum to confer passive protection against *N. meningitidis* bacteremia was tested in infant rats challenged ip, performed as described elsewhere [14]. The day before the challenge, freshly thawed bacteria were inoculated onto chocolate agar and were grown overnight at 37°C in 5% CO$_2$. On the morning of the challenge, several colonies were inoculated in Mueller-Hinton broth supplemented with 0.25% (wt/vol) glucose. After inoculation of the bacteria, to a starting $A_{620}$ of $\sim 0.1$, the test organism was grown for $\sim 2$ h with shaking, at 37°C in 5% CO$_2$, to an $A_{620}$ of $\sim 0.6$. After washing the bacteria twice in PBS containing 1% (wt/vol) BSA (PBS-BSA), the bacterial suspension was diluted in PBS-BSA, to contain $\sim 40,000$ cfu/mL. The protection and challenge were performed as follows: 5–7-day-old pups from litters of out-bred Wistar rats (Charles River) were randomly redistributed to the nursing mothers. Groups of 5–6 animals were treated ip at time 0 with different dilutions of test or control antisera or MAbs. Two hours later, the animals were challenged ip with 100 $\mu$L of $\sim 4 \times 10^7$ cfu of *N. meningitidis* group B or C bacteria. Eighteen hours after the bacterial challenge, blood specimens were obtained by puncturing the heart with a syringe containing $\sim 25$ U of heparin without preservative (American Pharmaceutical Partners). Aliquots of 1, 10, and 100 $\mu$L of blood were plated onto chocolate agar. The colony-forming units per milliliter of blood was determined after incubating the plates overnight at 37°C in 5% CO$_2$.

**Bacterial cell preparations.** Bacterial cells from the 7 strains were grown at 37°C to mid-log phase in Mueller-Hinton broth supplemented with 0.25% (wt/vol) glucose. The cells were harvested by centrifugation and were resuspended in SDS sample buffer (0.06 mol/L Tris HCl [pH 6.8], 10% [vol/vol] glycerol, 2% [wt/vol] SDS, 5% [vol/vol] 2-mercaptoethanol, and 10 $\mu$g/mL bromophenol blue). After 3 cycles of freezing on dry ice, thawing at room temperature, and vortexing, the samples were boiled at 100°C and were loaded onto the 10% SDS-PAGE gel, as described below.

**SDS-PAGE and Western blots.** Membrane preparations were analyzed by use of 10% SDS-PAGE, as described by Laemmli [15], and a Mini-Protein II electrophoresis apparatus (Bio-Rad). For Western blots, the gel was equilibrated with buffer (48 mmol/L Tris HCl, 39 mmol/L glycine [pH 9.0], and 20% [vol/vol] methanol) and transferred to a nitrocellulose membrane (Bio-Rad) by use of a Trans-Blot (Bio-Rad) semidyed electrophoretic transfer cell. The nitrocellulose membranes were blocked with 2% (wt/vol) nonfat milk in PBS containing 0.2% (wt/vol) sodium azide. Anti-rGNA2132 antisera was diluted in PBS containing 1% (wt/vol) BSA, 1% (wt/vol) Tween-20, and 0.2% (wt/vol) sodium azide. Bound antibody was detected by use of rabbit anti-mouse IgG+A+M (H+L)–alkaline phosphatase conjugate polyclonal antibody (Zymed) and Sigma Fast 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrate (Sigma).

**RESULTS**

**Binding of anti-rGNA2132 antisera to the surface of live N. meningitidis cells.** Figure 1 shows the results of experiments measuring binding of IgG antibody to 3 encapsulated group B strains: NZ394/98 (the strain from which the sequence of the recombinant protein was derived), 2996, and S3032. The pos-

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**Figure 1.** Binding of polyclonal anti–recombinant genome-derived neisserial antigen 2132 (rGNA2132)–HisTag antiserum to live encapsulated meningococcal B bacteria from strains NZ394/98, 2996, and S3032, as determined by indirect fluorescence flow cytometry. A, Positive control anti–capsular-specific murine monoclonal antibody SEAM 41 tested at 25 $\mu$g/mL. B, A 1:10 dilution of the negative control antiserum prepared from mice immunized with adjuvant alone (black area) and the corresponding anti-rGNA2132–HisTag antiserum (white area). C, A 1:250 dilution of the anti-rGNA2132–HisTag antiserum.
itive control anti-group B polysaccharide MAb (SEAM 41) binds to all 3 strains (figure 1A). Figure 1B shows the respective binding of the negative control antiserum from mice immunized with adjuvant alone (figure 1B, black area) and the corresponding binding of the antiserum prepared from mice immunized with rGNA2132. There is a significant increase in binding in the anti-GNA2132 antiserum, as demonstrated by a shift to the right, indicating higher fluorescence intensity (figure 1B, white area). The largest increase in binding was for strains NZ394/98 and S3032, and the smallest was for strain 2996. At a 1:250 dilution (figure 1C), the respective anti-rGNA2132 binding activity to each of these strains was similar to that when measured at the 1:10 dilution (figure 1B), a result suggesting that, at both dilutions, antibody is present in excess of the surface-accessible GNA2132 epitopes. As summarized in table 1, the anti-rGNA2132 surface-binding activity to each of the other 4 N. meningitidis test strains was significantly above that of the negative control antiserum.

Complement deposition on live encapsulated bacteria. Figure 2 shows experiments measuring the effect of anti-rGNA2132 antibody on deposition of human C3b and iC3b on live encapsulated bacteria of group B strains NZ394/98, 2996, and S3032, as measured by flow cytometry. In the presence of the complement source alone, there was no evidence of complement deposition on the 3 strains (figure 2A). The addition of a positive control mouse anticapsular MAb increased the percentage of bacteria showing strong immunofluorescence with the anti-C3c antisera (figure 2B). Evidence of complement deposition was not seen when the bacterial cells were incubated with the anticapsular MAb and the complement source that had been heat-inactivated to remove complement activity (figure 2C). As shown in figure 2D and 2E, there was a significant increase in complement deposition when the bacterial cells were incubated with the mouse anti-rGNA2132 antiserum and human complement, compared with that of cells incubated with complement and negative control antiserum from mice immunized with adjuvant alone. We found similar evidence of increased complement deposition on the bacterial surface of the remaining 4 strains when incubated with the anti-rGNA2132 antiserum and human complement (data not shown).

Anti-rGNA2132 bactericidal activity. Figures 3A and 3B show the percentage survival of representative group B strains when incubated for 1 h at 37°C with different dilutions of the anti-GNA2132 antiserum and human (figure 3A) or rabbit (figure 3B) complement. When incubated with human complement, there was no detectable bactericidal activity against strain NZ394/98, which contains the GNA2132 gene encoding the recombinant protein used for the vaccine, but there was a high level of bactericidal activity against strain S3446. In contrast, when incubated with rabbit complement, the anti-GNA2132 antiserum was bactericidal against both strains (figure 3B). The bactericidal activity was specific for anti-GNA2132 antibody, since there was no bactericidal activity when incubated with human or rabbit complement when tested against a mutant of...
Anti-GNA2132 Passive Protection

Figure 3. Survival of Neisseria meningitidis after incubation in the presence of different dilutions of anti–genome-derived neisserial antigen 2132 (GNA2132) antisera and 20% complement. A, Human complement. B, Rabbit complement. The test strains were NZ394/98 (▲), S3446 (●), and a mutant of strain S3446 in which gna2132 has been inactivated (○). NZ394/98 is the strain from which the gene for expressing recombinant GNA2132 used in the vaccine was cloned.

Table 1 summarizes the anti-GNA2132 bactericidal titers measured against each of the 7 N. meningitidis strains. When tested with human complement, the anti-GNA2132 antiserum was bactericidal against 2 strains (S3032 and S3446), which are susceptible to anti-GNA2132 bacteriolysis elicited by human complement, and strains 2996, 4243, 4335, and NZ394/98, which are resistant to bacteriolysis. The amino acid sequence for GNA2132 from the remaining resistant strain, 8047 (resistant to bacteriolysis elicited by human complement), is identical to that of GNA2132 from strain 2996, and, therefore, the GNA2132 sequence of 2996 is shown to be representative of the sequence of the protein from the 2 strains (DNA sequences and translated protein sequences for these strains have been deposited in GenBank [accession numbers AY315192-AY315196]). The ORF for gna2132 from strain NZ394/98 encodes a mature protein of 476 aa. On the basis of the inferred amino acid sequences from the 7 strains, there are segments of GNA2132 that are variable and other segments that are highly conserved. The latter include aa 1–87 in the amino terminus and aa 285–476 in the carboxyl-terminal half of the protein. GNA2132 from strain NZ394/98 contains a 63-aa segment beginning at residue 88 that is absent in all of the other strains tested except S3446. Evidently, the presence or absence of this segment is inconsequential for the ability of the anti-rGNA2132 antiserum to elicit bactericidal activity with human complement, since the peptide segment is present in strains NZ394/98 (resistant to bacteriolysis) and S3446 (susceptible to bacteriolysis) and is absent in strain S3032 (susceptible to bacteriolysis) (table 1). Evidently, the presence or absence of this segment is inconsequential for the ability of the anti-rGNA2132 antiserum to elicit bactericidal activity with human complement, since the peptide segment is present in strains NZ394/98 (resistant to bacteriolysis) and S3446 (susceptible to bacteriolysis) and is absent in strain S3032 (susceptible to bacteriolysis) (table 1). Also, bactericidal activity in the presence of human complement appears to be not related to amino acid sequence, since the sequence of strains S3446 (susceptible to bacteriolysis) and NZ394/98 (resistant to bacteriolysis) are nearly identical (7 differences/476 aa).

Expression of GNA2132 as determined by Western blot.

Differences between strains in anti-GNA2132-mediated bacteriolysis could result from differences in expression of the protein. The relative amounts of GNA2132 present in each of the strains were compared by Western blot (figure 5) after separation by SDS-PAGE. Equal amounts of protein from each strain were loaded onto the gel. By Coomassie staining alone, GNA2132 is not evident in the different lanes (data not shown). For strains S3446 and NZ394/98, the Western blot shows an
Figure 4. Predicted amino acid sequences of mature protein of genome-derived neisserial antigen 2132 (GNA2132) from different *Neisseria meningitidis* strains. Sequencing data for *N. meningitidis* strain 2996 were obtained from GenBank (accession no. AF226421). The amino acid sequence for GNA2132 from strain 8047 is identical to that of GNA2132 from strain 2996, and, therefore, the GNA2132 sequence for strain 2996 is shown as representative of the sequence of the protein from the 2 strains. + indicates strains that were positive for anti–recombinant GNA2132 bactericidal activity when tested with human complement (see table 1).

anti-rGNA2132–reactive band having an apparent mass of ~81 kDa (figure 5, lanes 6 and 8, respectively), but this band is absent in membranes from a mutant strain of S3446 in which the gene encoding GNA2132 has been inactivated (figure 5, lane 7). The positive control rGNA2132-HisTag prepared from the gene of strain NZ394/98 (figure 5, lane 9) migrates with a lower apparent mass than that of the protein expressed in *N. meningitidis*, because the recombinant protein lacks the lipid modification and a 7-aa segment at the N-terminus (see Materials and Methods). Cell preparations from strains 2996, 8047, 4243, 4335, and S3032 have an anti-rGNA2132–reactive band of ~70 kDa (figure 5, lanes 1–5, respectively). The lower apparent mass of GNA2132 in these 5 strains, compared with that of strains NZ394/98 and S3446, was expected, because the former strains lack the 63-aa segment that is predicted to be present in GNA2132 from strains NZ394/98 and S3446 (figure 4). Among the 5 strains shown that express the smaller GNA2132 proteins, the respective intensity of staining is approximately the same. The possible exception is the immunoreactive band from strain S3032 (susceptible to bacteriolysis) (figure 5, lane 5), which has a slightly lower intensity than that of the other 4 strains. Staining of GNA2132 from strains S3446 and NZ394/98 appears to be more intense, compared with the respective bands from the other 5 strains. The difference may reflect greater expression of GNA2132 by these strains or, more likely, greater reactivity of the antisera against the proteins expressed by strains NZ394/98 and S3446, since the detecting antibody was prepared against the rGNA2132 from NZ394/98, and both strains contain the 63-aa insertion segment and have nearly identical amino acid sequences. An immunoreactive band running at the dye front in figure 5 (lanes 5, 6, and 7; strains S3032, S3446, and S3446 GNA2132 knockout, respectively), was detected in whole-cell preparations but not in outer-membrane vesicle preparations; therefore, it is unlikely to be accessible on the surface of bacteria. Also, the anti-rGNA2132 antisera was bacitracid against strain S3446 but not against the mutant S3446 knockout (figure 5, lane 7). On the basis of the Western blot described above and the results of bacterial binding measured by flow cytometry, it appears that there are no significant differences in the amounts of GNA2132 expressed by the test strains.
GNA2132 was inactivated (lane 7), strains 2996 (lane 1), strains 2996 serial antigen 2132 (rGNA2132)–HisTag antiserum. Solubilized cells from strains developed with anti–recombinant genome-derived Neisseria meningitidis (lane 5), S3446 (lane 2), S3032 (lane 3), 4335 (lane 4), S3032 (lane 5), S3446 (lane 6), a mutant of S3446 in which the gene encoding GNA2132 was inactivated (lane 7), strain NZ39498 (lane 8), and 0.01 µg of recombinant GNA2132–HisTag protein encoded by the gene from strain NZ394/98 (lane 9). The recombinant protein migrates with a lower apparent mass than that of the protein expressed in strain NZ394/98, because the recombinant protein lacks the lipid modification and a 7-aa segment at the N-terminus. Mr, relative molecular mass.

**Western blot of solubilized whole cells from Neisseria meningitidis strains developed with anti–recombinant genome-derived neisserial antigen 2132 (rGNA2132)–HisTag antiserum.** Solubilized cells from strains 2996 (lane 1), 8047 (lane 2), 4243 (lane 3), 4335 (lane 4), S3032 (lane 5), S3446 (lane 6), a mutant of S3446 in which the gene encoding GNA2132 was inactivated (lane 7), strain NZ39498 (lane 8), and 0.01 µg of recombinant GNA2132–HisTag protein encoded by the gene from strain NZ394/98 (lane 9). The recombinant protein migrates with a lower apparent mass than that of the protein expressed in N. meningitidis, because the recombinant protein lacks the lipid modification and a 7-aa segment at the N-terminus. Mr, relative molecular mass.

**Passive protection by anti-rGNA2132 antiserum.** The ability of the anti-GNA2132 antiserum to confer passive protection against bacteremia was evaluated in infant rats challenged ip with meningococcal strains that were resistant to anti-GNA2132 bacteriolysis when tested with human complement. Although not shown in table 1, these strains were also resistant to bacteriolysis when tested with infant-rat complement (titers, ≈1:1:4). Table 2 summarizes anti-GNA2132 protective activity in rats challenged with group B strain 2996 (experiment 1) or group C strain 4243 (experiment 2). Strain 2996 is representative of a strain that showed relatively poor surface binding by the anti-GNA2132 antiserum, as measured by flow cytometry (figure 1), whereas strain 4243 showed stronger binding. In experiment 1, the positive control anticapsular MAb, SEAM 3, was completely protective against bacteremia caused by strain 2996. A 1:10 dilution of the anti-rGNA2132 antiserum also gave complete protection. Anti-GNA2132 antiserum dilutions of 1:50 and 1:250 did not prevent bacteremia, but the geometric mean colony-forming units per milliliter of the treated rats was ≈10-fold lower than that of negative control animals pretreated with a 1:5 dilution of antiserum obtained from mice immunized with adjuvant alone or pretreated with buffer alone (P < .05).

In experiment 2, a 1:10 dilution of the anti-GNA2132 antiserum completely protected rats challenged with group C strain 4243 from developing bacteremia, whereas a 1:3 dilution of the antiserum from mice immunized with adjuvant alone provided no protection. The protective activity of the mouse anti-GNA2132 antiserum in this experiment was similar to that of pooled serum samples obtained 1 month after immunization of 15 children, 4.1–4.9 years old, who were given meningococcal polysaccharide vaccine [16]. This age group is known to be protected by vaccination [17–20]. In other experiments (not shown), the anti-GNA2132 antiserum conferred passive protection against bacteremia in infant rats challenged with group B strains NZ39498 and 8047 and group C strain 4335, all of which were resistant to anti-GNA2132 bacteriolysis elicited by human or infant-rat complement.

**DISCUSSION**

The genome-sequencing projects for *N. meningitidis* group B strain MC58 and group A strain Z2492 and *N. gonorrhoea* strain FA1090 have advanced the identification of a number of novel meningococcal vaccine candidates [9, 21, 22]. Specifically, the genome sequences provided a means for identifying potential surface-exposed proteins that had not been identified by classic immunological or microbiologic approaches. Pizza et al. used the MC58 genome-sequence information to clone, express, and immunize mice with a large number of potential surface-exposed proteins, an approach they termed “reverse vaccinology” [23]. One of the vaccine candidates that emerged from this process is the putative lipoprotein GNA2132. In the present study, we investigated the vaccine potential of GNA2132, by characterizing the ability of murine anti-rGNA2132 antisera to bind to the bacterial surface, activate deposition of complement factors and bacteriolysis, and passively protect infant rats against meningococcal bacteremia.

By flow cytometry, anti-rGNA2132 antibody (gene from strain NZ394/98) showed binding at relatively large dilutions (titer, ≈1:250) to all 7 group B and C strains tested. These included strains whose GNA2132 amino acid sequences differed from that of the vaccine protein by lacking a 63-aa insertion segment and, at numerous positions in a highly variable segment, following the insertion segment. These results suggest that rGNA2132 can elicit antibodies that are cross-reactive with epitopes in the conserved segments at the N- and C-terminal ends of the protein. We also found that anti-rGNA2132 antibody activates deposition of human C3 complement factors on the surface of all 7 strains tested. In addition, all 7 strains were killed in the presence of anti-rGNA2132 and rabbit complement. However, only 2 strains, S3446 and S3032, were susceptible to anti-GNA2132 bactericidal activity in the presence of human complement. Thus, despite antibody binding to the bacterial surface and deposition of human complement factors, there are differences between strains in the ability of anti-
Table 2. Passive protection of infant rats challenged with *Neisseria meningitidis*.

<table>
<thead>
<tr>
<th>Experiment (challenge strain), antiserum</th>
<th>Dose per rat, µg, or serum dilution</th>
<th>Blood culture at 18 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. positive/total</td>
<td>cfu/mL, geometric mean, (10^3)</td>
</tr>
<tr>
<td>Experiment 1 (B:2996)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>7/7</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Adjuvant only</td>
<td>1:5</td>
<td>7/7</td>
</tr>
<tr>
<td>Anticapsular MAb (G2b)</td>
<td>2</td>
<td>0/7</td>
</tr>
<tr>
<td>Anti-GNA2132</td>
<td>1:10</td>
<td>0/7</td>
</tr>
<tr>
<td></td>
<td>1:50</td>
<td>7/7</td>
</tr>
<tr>
<td></td>
<td>1:250</td>
<td>7/7</td>
</tr>
<tr>
<td>Experiment 2 (C:4243)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>5/5</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Pools prepared from serum samples of children, 4 years old ((n = 15))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preimmunization</td>
<td>1:5</td>
<td>5/5</td>
</tr>
<tr>
<td>Postimmunization</td>
<td>1:7.5</td>
<td>0/5</td>
</tr>
<tr>
<td>Pools prepared from serum samples of immunized mice ((n = 6))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adjuvant only</td>
<td>1:3</td>
<td>5/5</td>
</tr>
<tr>
<td>Anti-GNA2132</td>
<td>1:10</td>
<td>0/5</td>
</tr>
</tbody>
</table>

**NOTE.** Infant rats, 5–7 days old, were treated intraperitoneally \((ip)\) with different doses of control monoclonal antibodies \((MAbs)\) or antisera. In experiment 2, control pools were prepared from serum samples obtained before and 1 month after immunization of 4-year-old children with meningococcal polysaccharide vaccine \([16]\). Two hours after treatment with antibody, the animals were challenged \((ip)\) with \(4.2 \times 10^9\) cfu of group B strain 2996 (experiment 1) or \(1 \times 10^4\) cfu of group C strain 4243 (experiment 2). Quantitative blood cultures were obtained 18 h later. For calculation of geometric mean colony-forming units per milliliter, animals with sterile cultures \((100 \mu L\) of blood) were assigned values of \(1\) cfu/mL. GNA2132, genome-derived neisserial antigen 2132.

\(^a\) Geometric mean vs. that of animals given negative control antiserum.

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rGNA2132 antibody to progress to bacteriolysis elicited by human complement. Others also have reported C3 deposition on meningococci without progressing to bacteriolysis \([24–26]\).

We did not identify the underlying mechanism for why some strains are susceptible and others are resistant to human complement–mediated bacteriolysis with anti-rGNA2132 antibody. Several factors that are known to contribute to resistance to serum observed in *Neisseria* strains do not seem to have played a role. These include the presence of capsule polysaccharide, sialylation of lipooligosaccharide \((LOS)\), and expression of class 3 PorB \([27]\). The latter 2 provide ligands for binding of factor H, an important regulator of the alternative complement system pathway that rapidly converts C3b to iC3b. All strains tested express capsule polysaccharide on the basis of binding to the bacterial surface and susceptibility to bacteriolysis by anticapsular MAbs. Both anti-GNA2132–susceptible \((53032\) and 53446) and –resistant \((2996, 8047, 4243,\) and 4335) strains express the L3,7,9 immunotype LOS and can be sialylated at the terminal Gal-\(\beta\)-1-4GlcNac residue. Finally, both susceptible strains express class 3 PorB, which would be expected to confer resistance to bacteriolysis if factor H binding played a role in blocking the progression to bacteriolysis. Despite the apparent lack of contribution of these known resistance factors, it is possible that other surface molecules expressed by the resistant strains may block progression from C3b to formation or insertion of a functionally active membrane attack complex by interfering with steps along the terminal complement-activation pathway. Apparently, this blocking is not present when human complement is activated by antibodies to other surface antigens, such as capsule polysaccharide or PorA \((table 1)\), or with rabbit complement and anti-GNA2132 antibody, since all 7 strains were susceptible to anti-GNA2132 bacteriolysis elicited by rabbit complement \((figure 3\) and \(table 1)\). Also, the respective higher anti-GNA2132 bactericidal titers measured with rabbit complement were not unexpected, since several previous studies have reported that rabbit complement substantially augments meningococcal bactericidal activity, compared with that measured with human complement \([28–30]\). Despite the absence of bactericidal activity with human or infant-rat complement, we demonstrated the ability of anti-GNA2132 antisera to confer passive protection in an infant-rat model of meningococcal bacteremia after challenge with each of the 5 resistant strains. These results are important because they demonstrate that anti-GNA2132 antibodies are ac-
ative against bacteria that are rapidly replicating in the blood stream, where antigenic expression may be different from that of organisms grown in vitro or on mucosal surfaces [31, 32]. By flow cytometry, we demonstrated that anti-GNA2132 antibody bound to the bacterial cell surface and activated deposition of complement factors on the bacterial surface. Both C3b and the Fc portion of IgG antibody are known ligands for interacting with receptors on phagocytic cells and, thus, can serve as opsonins to enhance uptake of bacteria. Since there are only 2 known mechanisms of protection against meningococcal bacteremia—complement-mediated bactericidal activity and opsonophagocytic activity—in the absence of bactericidal activity, the most likely mechanism of protection is opsonophagocytosis.

Although data on additional strains are needed, the ability of rGNA2132 to elicit antibody in mice that is both reactive with heterologous proteins expressed by several group B and C strains and protective in the infant-rat challenge model of C3b and the Fc portion of IgG antibody are known ligands for interacting with receptors on phagocytic cells and, thus, can serve as opsonins to enhance uptake of bacteria. Since there are only 2 known mechanisms of protection against meningococcal bacteremia—complement-mediated bactericidal activity and opsonophagocytic activity—in the absence of bactericidal activity, the most likely mechanism of protection is opsonophagocytosis.

Although data on additional strains are needed, the ability of rGNA2132 to elicit antibody in mice that is both reactive with heterologous proteins expressed by several group B and C strains and protective in the infant-rat challenge model of meningococcal bacteremia shows that GNA2132 is a promising vaccine candidate for prevention of meningococcal disease.

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