Identification of Vaccine Candidates Against Serogroup B Meningococcus by Whole-Genome Sequencing

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Neisseria meningitidis is a major cause of bacterial septicemia and meningitis. Sequence variation of surface-exposed proteins and cross-reactivity of the serogroup B capsular polysaccharide with human tissues have hampered efforts to develop a successful vaccine. To overcome these obstacles, the entire genome sequence of a virulent serogroup B strain (MC58) was used to identify vaccine candidates. A total of 350 candidate antigens were expressed in Escherichia coli, purified, and used to immunize mice. The sera allowed the identification of proteins that are surface exposed, that are conserved in sequence across a range of strains, and that induce a bactericidal antibody response, a property known to correlate with vaccine efficacy in humans.

Meningococcal meningitis and sepsis are devastating diseases that can kill children and young adults within hours despite the availability of effective antibiotics. The diseases are caused by Neisseria meningitidis, a Gram-negative, capsulated bacterium that has been classified into five major pathogenic serogroups (A, B, C, Y, and W135) on the basis of the chemical composition of distinctive capsular polysaccharides (1). The reported annual incidence of meningococcal disease varies from 0.5 to 10 per 100,000 persons (2, 3). However, during epidemics the incidence can rise above 400 per 100,000 (2, 3). The case fatality rate ranges from 5% to 15%, and up to 25% of survivors are left with neurological sequelae (4). Recently, 2600 and 5606 cases were reported annually in the United States and Europe, respectively (4).

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In the 1960s, vaccines consisting of purified polysaccharide antigens were developed against four (A, C, Y, and W135) of the five pathogenic serogroups (1). These vaccines are highly effective in adults but are not efficacious in infants and young children, the age groups mostly exposed to disease. Second-generation glycoconjugate vaccines that are also effective in infants and children are now in the later phases of development (2, 5). Currently, there are no vaccines available for prevention of serogroup B N. meningitidis (MenB) disease, which is responsible for 32% of all meningococcal disease in the United States and for 45% to >80% of the cases in Europe (6). The use of capsular polysaccharide as the basis of a vaccine for prevention of MenB diseases has been problematic. The MenB capsular polysaccharide is identical to a widely distributed human carbohydrate [α(2→8)N-acetyl glucosamine acid or polysialic acid], which, being a self-antigen, is a poor immunogen in humans. Furthermore, use of this polysaccharide in a vaccine may elicit autoantibodies (7). An alternative approach to vaccine development is based on surface-exposed proteins contained in outer membrane vesicles (OMVs). These vaccines have been shown both to elicit serum bactericidal antibody responses and to protect against developing meningococcal disease in clinical trials (8). However, the limitation of OMV vaccines is that the major protein antigens show sequence and antigenic variability and, although they induce protective antibodies against the homologous strain, they fail to induce protection against heterologous strains (9). With the recent exception of NspA (10), all the surface-exposed proteins described during the past three decades have in common the drawback of antigenic variability (2).

In order to identify potential vaccine candidates, we determined the genome sequence of the virulent strain MC58 [see (11)]. While the sequencing project was in progress, unassembled DNA fragments were analyzed to identify open reading frames (ORFs) that potentially encoded novel surface-exposed or exported proteins (12).

We identified 570 such ORFs and, by means of the polymerase chain reaction (PCR), we amplified and cloned the DNA sequences of these hypothetical genes in Escherichia coli to express each polypeptide as either His-tagged or glutathione S-transferase (GST) fusion proteins (13). We obtained successful expression with 350 ORFs (61%). More specifically, 70 predicted lipoproteins, 96 predicted periplasmic proteins, 87 predicted inner membrane proteins, and 45 predicted outer membrane proteins; there were 52 proteins with uncertain prediction. Proteins with more than one hydrophobic trans-membrane domain had the highest rate of expression failure. The recombinant proteins were purified and used to immunize mice (13). Immune sera were then tested in enzyme-linked immunosorbent assay (ELISA) and fluorescence-activated cell sorter (FACS) analyses to detect proteins that were present on the surface of a set of MenB strains selected to represent the diversity of invasive strains within the natural population of this species (14). In addition, we tested the immune sera for bactericidal activity (14) because this assay correlates with protection in humans (15). Of the 85 proteins found to be strongly positive in at least one of the above assays, we selected for further studies seven representative proteins (genome-derived Neisseria antigens; GNA) that were positive in all three assays (Table 1) and whose genes were not predicted to be phase variable (11). Each of the proteins raised an immune response that induced complement-mediated bactericidal activity. In the case of proteins GNA33 and GNA2132, the resulting bactericidal titers were similar in magnitude to that induced by OMV, which is known to confer protection in humans against homologous strains (9).

To test the suitability of these proteins as candidate antigens for conferring protection against different MenB strains and not just against the homologous strain, we used a collection of strains isolated worldwide and
over many years to investigate whether the new candidate molecules were conserved and accessible to antibodies. Our aim was to select strains representative of the diversity found in natural populations of MenB. We used a phylogenetic tree from 107 strains constructed by multilocus enzyme electrophoresis (MLEE) and validated by multilocus sequence typing (MLST) to select 22 representative, disease-associated MenB strains (16–19) (Fig. 1A). In addition, in the analysis we included three strains of *N. meningitidis* serogroup A; two strains of serogroup C; one strain each of serogroups Y, X, Z, and W135; three strains of *Neisseria gonorrhoeae*; and one strain each of *Neisseria cinerea* and *Neisseria lactamica* (19). PCR products from the seven genes were detected in each of the 31 strains of *N. meningitidis* and most were also found in the *N. gonorrhoeae* isolates. In *N. lactamica* or *N. cinerea*, PCR was often negative, but we detected similar sequences by Southern blotting (Table 2). We determined the nucleotide sequence of the seven genes and *porA* in all the *N. meningitidis* and *N. gonorrhoeae* strains (Fig. 2) (GenBank accession numbers: AF226325–AF226572, *N. meningitidis*; AF235143–AF235159, *N. gonorrhoeae*). *PorA* was included in the analysis because it is a well-characterized membrane-associated protein that displays sufficient sequence diversity (2, 20) to render it unsuitable as an effective vaccine. As expected, *PorA* showed regions of substantial amino acid sequence divergence in MenB as well as in the other meningococcal serogroups (Fig. 2) (19). Among the antigens, GNA992 and GNA2132 had many hypervariable regions, located mostly in the amino-terminal half of the molecules, which suggests that these proteins, like *PorA*, might induce strain-specific immunity. In marked contrast, GNA33, GNA1162, GNA1220, GNA1946, and GNA2001 showed 99.2% ± 0.7%, 99.7% ± 0.7%, 99.7% ± 0.3%, 99.4% ± 0.3%, and 99.7% ± 0.3% average amino acid identity to the MCS8 sequence, respectively, within the 31 *N. meningitidis* strains analyzed. The results suggest that these proteins may induce immunity against most strains of MenB and, possibly, against the other pathogenic strains of *N. meningitidis*. GNA33, GNA1162, GNA1220, and GNA1946 showed 95.8% ± 0.2%, 96.5% ± 0.4%, 99% ± 0.0%, and 97.6% ± 0.0% identity, respectively, to *N. gonorrhoeae*; therefore, they are also candidate vaccine antigens against this pathogen.

To further characterize these target antigens as vaccine candidates, we studied surface expression by immunoblotting outer membrane preparations and we studied accessibility of antibodies with encapsulated bacteria in a whole-cell ELISA (14). These data provided evidence that each of the seven proteins was expressed and available for analysis.

![Dendrogram showing genetic relationship among 107 N. meningitidis strains based on MLST analysis of six gene fragments (adapted from Maiden et al. (16)]. The dendrogram was used to select strains representative of serogroup B meningococcus population (arrows). Five additional strains, for which genetic assignment to hypervirulent lineages was independently determined by Wang et al. (18), Seiler et al. (17), and Virji et al. (19), are superimposed in the dendrogram and indicated by asterisks. In addition to the 22 strains of MenB, three strains of MenA, two strains of MenC, and one strain each of Men Y, X, Z, and W135 were used. These are indicated with a boldface letter before the name. When phylogenetic data were not available, the strains were reported above the figure outside the tree. The hypervirulent clusters ET-5, ET-37, and IV-1 are indicated by colored vertical bars. (A) Dendrogram of *N. meningitidis* strains obtained from the conserved genes reported in Table 2. Phylogenetic analysis based on the new genes provided a dendrogram that clusters the hypervirulent strains, in agreement with the results of MLEE and MLST reported in (A). Colored and gray bars indicate strains that cluster with 100% bootstrap support in agreement with MLST analysis. Numbers at the base of each node are bootstrap scores (only those >80% are reported). Gene sequences from different strains were aligned with the program PileUp of the GCG package. Phylogenetic analysis was done with the neighbor-joining algorithm (24) as implemented in the program NEIGHBOR of the PHYLIP package. Pairwise distances were calculated by using the Kimura-two parameter (25) on the 31 *N. meningitidis* strains. We excluded the NH2-terminal region of GNA992, the entire GNA2132, and the tandemly repeated regions of GNA2001 from the analysis. We allowed a total of 1000 bootstrap replicates to evaluate the level of support. We confirmed clustering of the hypervirulent strains by maximum parsimony analysis.
tibody binding in the presence of the polysaccharide capsule coating the bacteria. Surface exposure also was determined by FACS analysis, measuring binding of antibodies to bacterial strains whose capsule had been permeabilized by treatment with ethanol (14). We found the seven proteins to be expressed and surface exposed in all 31 N. meningitidis strains tested. The hyperimmune sera prepared against the conserved proteins GNA33 and GNA1946 recognized equally the homologous MenB strain 2996 (13) and the heterologous MenB strain BZ232 (Fig. 3) (17). Conversely, antiserum prepared against the OMV from strain 2996, which is known to confer strain-specific protection (9), bound strongly to the homologous strain but weakly to the heterologous strain. As expected, antiserum against GNA33 and GNA1946 showed complement-mediated bacteriolysis against strains 2996 and BZ232. Both sera also had bacterial activity against three unrelated, heterologous strains of MenB, for which a common source of suitable human complement was available (14).

The finding of several conserved surface-exposed antigens in N. meningitidis is surprising, because in three decades of studies, with one exception (10), only antigenically variable proteins had been described. To gain more information about the genetic diversity of the newly discovered conserved proteins, we evaluated the frequency of recombination of their genes by means of the homoplasy test (19, 21). We obtained an average value of 0.11, a result indicating a low level of recombination, similar to that previously obtained for Neisseria housekeeping genes (19). The result was substantiated by a phylogenetic analysis with the neighbor-joining algorithm. The outcome of this analysis was a dendrogram clustering the hypervirulent strains of complexes ET-37, ET-5, and subgroup IV-1 (Fig. 1B), in agreement with the dendrogram generated by MLST and MLEE through housekeeping genes (Fig. 1A) (16–18).

Thus, the newly identified surface proteins, apart from being surface exposed and accessible to antibodies, are surprisingly conserved, as previously observed in housekeeping proteins. In the case of protein GNA33, the presence of a predicted enzymatic activity of importance in peptidoglycan metabolism may explain the conservation of this gene (22). Alternatively, because the new proteins are less abundant than major outer membrane proteins (23), they may be poorly immunogenic during infection and therefore subject to lower selection pressures from the host’s immune system. Overall, these data confirm that the newly discovered proteins behave differently from the other surface antigens known so far and are likely to confer...
Properties of the proteins.

<table>
<thead>
<tr>
<th>Antigen (length, amino acids)</th>
<th>Remarks/similarities*</th>
<th>FACS</th>
<th>ELISA</th>
<th>Serum bactericidal activity (SBA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GNA33 (441) Lipoprotein/similar to E. coli membrane-bound lytic transglycosylase A (MltA) of E. coli and of Synecocystis sp. (22)</td>
<td>++ + + ++ †</td>
<td>13,000</td>
<td>1/16,000†</td>
<td></td>
</tr>
<tr>
<td>GNA992 (591) Outer membrane protein/similar to Hsf and Hia of Haemophilus influenzae and FhaB of Bordetella pertussis (26)</td>
<td>++ +</td>
<td>2,750</td>
<td>1/256</td>
<td></td>
</tr>
<tr>
<td>GNA1162 (215) Lipoprotein/no significant similarities</td>
<td>++</td>
<td>1,270</td>
<td>1/4</td>
<td></td>
</tr>
<tr>
<td>GNA1220 (315) Membrane protein/contains a stomatin-like domain</td>
<td>+</td>
<td>1,000</td>
<td>1/256</td>
<td></td>
</tr>
<tr>
<td>GNA1946 (287) Lipoprotein/similar to HlpA of H. influenzae, belongs to the NlpA family of lipoproteins (27)</td>
<td>+ + +</td>
<td>13,100</td>
<td>1/32</td>
<td></td>
</tr>
<tr>
<td>GNA2001 (251) Outer membrane protein/similar to P60 invasion-associated extracellular proteins (28)</td>
<td>++</td>
<td>500</td>
<td>1/512</td>
<td></td>
</tr>
<tr>
<td>GNA2132 (488) Lipoprotein/lowl similarity to transferrin binding proteins</td>
<td>+ +</td>
<td>1,700</td>
<td>1/16,000</td>
<td></td>
</tr>
<tr>
<td>GST§ OMV‡</td>
<td></td>
<td>+ +</td>
<td>260,000</td>
<td>1/32,000</td>
</tr>
</tbody>
</table>

*Homolog searches have been performed against nonredundant protein databases by means of the BLAST algorithm (National Center for Biotechnology Information). Hits with an E score of < 10 to an assigned biological role are reported. † ++ + + + + indicate no difference with preimmune serum or differences in fluorescence of 0.5, 1, 1.5, and 2 or more orders of magnitude, respectively. ‡ Serum bactericidal cell activity (SBA) was evaluated with pooled baby rabbit serum as complement source. Tilters were expressed as the reciprocal of the serum fluorescence of 0.5, 1, 1.5, and 2 or more orders of magnitude, respectively.

Table 2. Presence of genes in Neisseria.

<table>
<thead>
<tr>
<th>N. meningitidis</th>
<th>N. lactamica (1 strain)</th>
<th>N. cinerea (1 strain)</th>
<th>N. gonorrhoeae (3 strains)</th>
</tr>
</thead>
<tbody>
<tr>
<td>gna33</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>gna992</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>gna1162</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>gna1220</td>
<td>†</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>gna1946</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>gna2001</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>gna2132</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>porA</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*In strains NC6/88 and NGF26 the start codon is 222 bases downstream from the starting codon in the other strains. † ++ + + + + indicates a negative PCR but positive Southern blotting. ‡ In strain B2133 a deletion of 31 nucleotides causes a frameshift in this gene.

In addition to proving the potential of the genomic approach, by identifying highly conserved proteins that induce bactericidal antibodies, we have provided candidates that will be the basis for clinical development of a vaccine against an important pathogen. This vaccine is likely to elicit cross protection not only against group B N. meningitidis but also against other serogroups and species of pathogenic Neisseria.

References and Notes
12. Sequence analysis: A primary screening for coding capacity of the N. meningitidis genome sequence (11) was carried out on DNA segments or contigs with databases and computer programs included in the Wisconsin package version 10.0, Genetics Computer Group (GCG), Madison. Wi. First we used BLASTX to identify two classes of DNA segments with potential coding regions. The class of ORFs coding for known cytoplasmic functions was not further investigated, whereas the other classes of coding regions were selected for further analyses. A second screening step aimed at identifying putative proteins with cellular localization spanning from the outer membrane to the outside of the bacterium was applied. BLAST, FASTA, MOTIFS, FINDPATTERNS, and PSORT (http://psort.nibb.ac.jp), as well as the ProDom, Pfam, and Blocks databases, were used to predict features typical of surface-associated proteins such as transmembrane domains, leader peptides, homologs to known surface proteins, lipoprotein signature, outer membrane anchoring motives, and host cell binding domains such as RGD. The screening yielded 570 ORFs with the desired features, which were screened for conservation against N. meningitidis serogroup A and N. gonorrhoeae partial genome sequences available at The Sanger Centre (http://www.sanger.ac.uk/Projects/N.meningitidis/) and the Advanced Center for Genome Technology at The University of Oklahoma (http://www.genome.ou.edu/gono.html), respectively. This analysis indicated that 98.6% of the selected N. meningitidis ORFs are also found and conserved in serogroup A and 95.3% are found and conserved in N. gonorrhoeae.
13. ORFs were amplified by PCR on chromosomal DNA from strain 2996 [P. van der Ley and J. T. Poolman, Infect. Immun. 60, 3156 (1992)], with synthetic oligonucleotides used as primers. The amplified DNA fragments were cloned into pET-Z1b vector (Novagen) and pCGR-KG vector (K. L. Guan and J. E. Dixon, Anal. Biochem. 192, 262 (1991)] to express the proteins as COOH-terminus His-tagged and NH2-terminus GST fusions, respectively. We evaluated expres- sion of recombinant proteins according to the appearance of bands in SDS-polyacrylamide gel elec- trophoreses. We purified fusion proteins by affinity chromatography on Ni2+-conjugated chelating fast-flow Sepharose (Pharmacia) or glutathione-Sepha- rose 4B resin (Pharmacia). Proteins expressed as insoluble inclusion bodies were solubilized with guan- dinium and/or urea and renatured after purification [J. E. Colligan, B. M. Dunn, H. L. Rineh, D. W. Speiches, P. T. Wingfield, Eds., Current Protocols in Protein Science (Wiley, New York, 1997), pp. 6.0.1–6.7.14, 9.4.1–9.4.16]. We prepared OMVs by Sarkosyl extraction as described [R. L. Davies, R. A. Wall, S. P. Sorrell, J. Immunol. Methods 134, 215 (1991)]. We then mixed 20 μg of each purified protein or 2996 OMV with Freund’s adjuvant and used it to immunize CD1 mice at days 1, 21, and 35. Blood samples were taken on day 34 and 49.
14. Sera analysis: Antibody titers were determined by ELISA on microtiter plates coated with the capsulated strains inactivated by treatment with 0.025% para-
gridlock, an HLH Gene Required for Assembly of the Aorta in Zebrafish
Tao P. Zhong,* Michael Rosenberg, † Manzoor Ali-P K. Mohideen, ‡ Brant Weinstein,* Mark C. Fishman†

The first artery and vein of the vertebrate embryo assemble in the trunk by migration and coalescence of angioblasts to form endothelial tubes. The gridlock (grl) mutation in zebrafish selectively perturbs assembly of the artery (the aorta). Here it is shown that grl encodes a basic helix-loop-helix (bHLH) protein belonging to the Hairy/Enhancer of the split family of bHLH proteins. The grl gene is expressed in lateral plate mesoderm before vessel formation, and thereafter in the artery and not in the vein. These results suggest that the arterial endothelial identity is established even before the onset of blood flow and implicate the grl gene in assignment of vessel-specific cell fate.

Arteries and veins are morphologically and functionally very distinct. For example, arterioles deliver oxygenated blood at high pressure, whereas veins serve as capacitance vessels for blood return. Some of the morphological differences may be imposed after, and depend upon, onset of function. However, a complete vascular loop, composed of the trunk dorsal aorta and posterior cardinal vein, is needed to accommodate the output of the first heartbeat. These simple tubes of endothelium form by local aggregation of angioblasts, a process termed vasculogenesis (1). Neither mutations nor molecular markers have revealed whether there are arterial-venous distinctions between angioblast progenitors. In the mouse, ephrinB2 is selectively expressed on the arteries and EphB3 and EphB4 on the veins, but this occurs after vasculogenesis (2). Furthermore, mutation of ephrinB2 does not affect vasculogenesis, although it does disrupt later vessel formation and remodeling, a process termed angiogenesis (2).

The gridlock mutation (grl1143) was originally isolated in a large-scale chemical mu-

R E P O R T S

formaldehyde or with the noncapsulated M7 strain [D. S. Stephens, J. S. Swartley, S. Kathariou, S. A. Morse, Infect. Immun. 59, 4097 (1991)]. Immunoglobulin tethers were expressed as the reciprocal of serum dilution that gave an A500 value of 0.4 above the background. Immunoblots of purified proteins, OMVs, or total cell extracts with 15% polyacrylamide gels and 1:200 sera dilutions. We evaluated serum bactericidal activity against strains 2996 and BZ232, as described [C. C. A. Peeters et al., Vaccine 17, 2702 (1999)], with pooled baby rabbit serum used as complement source [Cedar Lane]. The serum bactericidal titers were reported as the reciprocal of the serum dilution yielding ≥50% bacterial killing. We tested serum bactericidal activity with human complement, as described [R. T. M. Ochman, J. Infect. Dis. 172, 1279 (1995)], against strains 8047 (obtained from W. Zollinger), M986 [D. A. Caugant et al., J. Bacteriol. 169, 2781 (1987)], and NGP165 [17]—the strains in our collection that could be tested with our human complement source. The noncapsulated M7 strain or cap-

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The first artery and vein of the vertebrate embryo assemble in the trunk by migration and coalescence of angioblasts to form endothelial tubes. The gridlock (grl) mutation in zebrafish selectively perturbs assembly of the artery (the aorta). Here it is shown that grl encodes a basic helix-loop-helix (bHLH) protein belonging to the Hairy/Enhancer of the split family of bHLH proteins. The grl gene is expressed in lateral plate mesoderm before vessel formation, and thereafter in the artery and not in the vein. These results suggest that the arterial endothelial identity is established even before the onset of blood flow and implicate the grl gene in assignment of vessel-specific cell fate.