Vaccination with Attenuated *Neisseria meningitidis* Strains Protects against Challenge with Live Meningococci

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Meningococcal disease is a life-threatening infection caused by *Neisseria meningitidis*. Currently, there are no vaccines to prevent infection with serogroup B *N. meningitidis* strains, the leading cause of meningococcal meningitis in Europe and North America. Here we describe the construction and characterization of two attenuated serogroup B *N. meningitidis* strains, YH102 (MC58Δ*asia* Δ*rfaF*) and YH103 (MC58Δ*asia* Δ*metH*). Both strains are markedly attenuated in their capacity to cause bacteremia in rodent models and have a reduced ability to survive in a human whole-blood assay. Immunization of adult mice with these strains leads to the development of bactericidal antibodies and confers sterilizing protection against challenge with homologous live bacteria. Furthermore, we show that the strains confer protection against infection by other serogroups. Use of the attenuated strains in animals with gene knockouts or after depletion of immunological effectors could be used to define the basis of protection, and human volunteer studies could be undertaken to examine the immune response following exposure to this important human pathogen.

Neisseria meningitidis causes purulent meningitis and septic shock (2). In developed countries, *N. meningitidis* is the leading cause of bacterial meningitis and remains an important public health problem (27). The nonspecific early clinical signs and fulminant course of meningococcal infection mean that therapy is often ineffective. Therefore, vaccination is considered the most effective strategy to diminish the global disease burden caused by this pathogen (5). Existing vaccines to prevent infections caused by serogroup A, C, W135, and Y *N. meningitidis* are based on the polysaccharide capsule located on the surface of the bacterium (1, 20, 21). Progress toward a vaccine against serogroup B infections has been more difficult as its capsule, a homopolymer of α(2-8)-linked sialic acid, is a relatively poor immunogen in humans because it shares epitopes expressed on a human neural cell adhesion molecule, N-CAM1 (6). Indeed, generating immune responses against the serogroup B capsule might actually prove harmful. Thus, there remains a need for new vaccines to prevent serogroup B *N. meningitidis* infections.

An alternative approach for combating bacterial infection is the development of live attenuated vaccines. Notable examples of live attenuated bacterial vaccines include the licensed *Salmonella enterica* serovar Typhi vaccine Ty21a, two recombinant typhoid strains that are in clinical development, and *Mycobacterium bovis* BCG (13, 29, 38). Multivalent live attenuated vaccines can also be used to circumvent limitations imposed by antigenic variation. For example, Mel and colleagues derived

streptomycin-dependent strains of the most prevalent *Shigella* serotypes (23, 24). When administered as a multivalent vaccine, the strains conferred protection against dysentery caused by the serotypes present in the vaccine.

In this study, we describe the selection and construction of attenuated strains of serogroup B *N. meningitidis* based on a recent analysis of genes required for systemic infection (28). Our aim was to determine whether vaccination with attenuated strains elicits protective immunity against meningococcal challenge.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth. The bacterial strains and plasmids used in this study are given in Table 1. *N. meningitidis* was grown on brain heart infusion (BHI) medium with Levanthal's supplement in the presence of 5% CO₂ at 37°C. *Escherichia coli* was propagated in Luria Bertani medium. For *E. coli* and *N. meningitidis*, antibiotics were added at the following concentrations: kanamycin at 50 and 75 μg/ml, respectively, and erythromycin at 200 and 2 μg/ml, respectively.

DNA extraction, PCRs, and Southern analysis. Bacterial genomic DNA was extracted by standard methods. PCRs were performed with *Taq* polymerase (Gibco BRL) in a Perkin-Elmer Cetus 9600 Thermal cycler. The oligonucleotide pairs NG364 (5'-AGCGGGATTGGGCGGAAGATTACC-3') with NG365 (5'-AGCAATCGGTGCAAAATGCCGAAG-3') and NG366 (5'-AGCCTGTTGCCTAACTGTTGCTG-3') with NG367 (5'-ACCCAGTTTGGCTTCAGAGGCTCG-3') were used to amplify the defective *rfaF* and *metH* alleles from mutants 3E7 and 15G9, respectively.

Protection model and immunization schedule. Six-week-old adult mice (BALB/c; Harlan) were vaccinated on days 0 and 21 with the attenuated strains given by the intraperitoneal (i.p.) route. Bacteria were grown overnight on a solid medium and then harvested into phosphate-buffered saline (PBS). The number of CFU was estimated by measuring the optical density at 260 nm (OD₂₆₀) of a lysate of the suspension in 1% sodium dodecyl sulfate–0.1% NaOH (an OD of 1.8 is equivalent to 10⁹ CFU/ml), and the number of viable bacteria was confirmed by plating to solid media. Groups of animals were immunized with recombinant refolded PorA from strain MC58 (kindly provided by Microscience Ltd.). For the live challenge, bacteria were administered to animals in BHI with 0.5% (wt/vol) iron dextran (Sigma, Poole, United Kingdom). Animals were observed at least twice daily for 72 h after inoculation. Distressed animals (showing reduced movement, labored breathing, and ruffled fur) were eutha-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
<i>N. meningitidis</i> strains		
MC58	Clinical serogroup B <i>N. meningitidis</i> , isolate ET-5	31
C311	Clinical serogroup B <i>N. meningitidis</i> isolate ET-5	34
BZ169	Clinical serogroup B <i>N. meningitidis</i> isolate ET-5	25
1000	Clinical serogroup B <i>N. meningitidis</i> isolate	25
NGP165	Clinical serogroup B <i>N. meningitidis</i> isolate ET-37	25
90/18311	Clinical serogroup C <i>N. meningitidis</i> isolate ET-37	25
A22	Clinical serogroup W135 <i>N. meningitidis</i> isolate	25
205900	Clinical serogroup A <i>N. meningitidis</i> isolate IV-1	25
15G9	C311 Δ <i>metH</i>	28
YH100	MC58 Δ <i>rfaF</i>	This study
YH101	MC58 Δ <i>metH</i>	This study
YH102	MC58 Δ <i>rfaF</i> Δ <i>siaD</i>	This study
YH103	MC58 Δ <i>metH</i> Δ <i>siaD</i>	This study
<i>E. coli</i> strain		
DH5 α	F' <i>endA1 supE44 thi-1 hsdR17</i> ($r_R^- m_R^+$) <i>recA1 gyrA relA1</i> Δ (<i>lacIZYA-argF</i>) <i>U169, deoR</i> (ϕ 80d <i>lac</i> Δ [<i>lacZ</i>] <i>M15</i>)	Gibco BRL
Plasmids		
pIP10	<i>siaD</i> Δ <i>ery</i> in puC18	35
pCR2.1 Topo	TA cloning vector	Invitrogen
pYH70	<i>rfaF</i> with a kanamycin resistance cassette in pCR2.1 Topo	This study
pYH71	<i>rfaF</i> with a kanamycin resistance	This study

nized by CO₂ narcosis. All challenges were performed at least 2 weeks after the last immunization. Bacteremia was assessed by collecting 5 μ l of blood from a tail vein bleed, and the survival of animals was compared by a one-tailed Student's *t* test.

WBA. The whole-blood assay (WBA) was performed as described previously (15). Briefly, *N. meningitidis* was grown to mid-log phase by inoculation from an overnight culture into fresh GC (Difco Laboratories, Moseley, Surrey, United Kingdom) medium supplemented with IsoVitalX and incubated for 4 h at 37°C in 6% CO₂. The number of CFU was enumerated as above, and 1 ml of heparinized human blood was inoculated with 10⁷ CFU and rocked at 20 rpm for two hours. Samples (10- μ l) were removed before incubation and at 30-min intervals thereafter. The number of viable organisms was determined, and results were expressed as the percentage of organisms surviving in comparison with the initial count.

Immunologic assays. For whole-cell enzyme-linked immunosorbent assays (ELISAs), heat-killed cells were coated onto wells of microtiter dishes (Maxisorb; Nunc) overnight at 4°C, washed with PBS–0.1% Tween 20, and then incubated with dilutions of sera for 2 h at room temperature; preimmune sera were used as negative controls. After the wells were washed, a horseradish peroxidase-conjugated goat anti-mouse antibody (DAKO) was added at a dilution of 1:1,000 in PBS–0.1% Tween 20, and the mixture was incubated for a further 2 h. After the wells were washed, the substrate (*o*-phenylenediamine dihydrochloride; Sigma) was added and the absorbance was read at 492 nm.

The serum bactericidal assay (SBA) titer was calculated as the reciprocal serum dilution yielding $\geq$ 50% bacterial killing. Approximately 250 CFU of strain MC58 were incubated with baby rabbit (for strains MC58 and 205900) or human (for 90/18311) complement with dilutions of heat-treated serum for 1 h at 37°C. The number of viable bacteria at the start and at the end of the incubation was determined by plating to solid media. All assays were performed in duplicate.

RESULTS

Selection and construction of mutations for inclusion in attenuated strains. Three genes were chosen for deletion in two attenuated strains; the genes had previously been identified in an analysis of serogroup B *N. meningitidis* to identify bacterial factors required for septicemic disease (28).

siaD (mutant 5E5) encodes the sialyltransferase required for polymerization of sialic acid residues in the serogroup B cap-

sule (7). *siaD* was inactivated in both vaccine strains. *rfaF* (mutant 23A10) is part of an operon involved in lipopolysaccharide (LPS) biosynthesis and encodes an ADP-heptose heptosyltransferase (16, 17). The *metH* (mutant 15G9) product is predicted to catalyze the final step in methionine biosynthesis.

To construct the mutant strains in an MC58 genetic background, the defective *rfaF* and *metH* alleles were amplified by PCR from the corresponding C311 mutants (Table 1) with primer pairs NG364/NG365 and NG366/NG367, respectively. PCR products of the predicted length were purified and then ligated into pCR 2.1 Topo, yielding pYH70 (for *rfaF*) and pYH71 (for *metH*), and the identity of the inserts was verified by nucleotide sequencing. The plasmids were linearized by restriction endonuclease digestion and then used to transform MC58 to kanamycin resistance, producing YH100 (MC58 Δ *rfaF*) and YH101 (MC58 Δ *metH*). Next, YH100 and YH101 were both transformed with plasmid pIP10, which harbors a deleted copy of *siaD* containing *ermC* (35), encoding resistance to erythromycin. This process resulted in YH102 (MC58 Δ *siaD* Δ *rfaF*) or YH103 (MC58 Δ *siaD* Δ *metH*), respectively. Southern analysis confirmed that the constructs had integrated by homologous recombination (data not shown).

Loss of *rfaF*, *metH*, and *siaD* attenuates *N. meningitidis*. The mutations included in strains YH102 and YH103 had been identified by their role in attenuating another serogroup B strain in the infant rat model (28). To establish whether the mutations also attenuate strain MC58 in a murine model, mice were challenged with YH102, YH103, or MC58, and their survival was monitored. Most animals receiving 10⁴ CFU of MC58 remained healthy following challenge, with a few mice developing ruffled fur between 12 and 24 h after infection. However, after challenge with 10⁶ CFU, animals became sick within 12 h and showed signs of distress, with most mice suc-

FIG. 1. (A) Survival of animals following i.p. challenge with wild-type *N. meningitidis* MC58. (B) YH102 (MC58 Δ siaD Δ rfaF) is markedly attenuated compared with the wild type; similar results were obtained for YH103 (MC58 Δ siaD Δ metH) (data not shown). (C) Bacteremia following challenge with MC58, YH102, and YH103. Results are shown for individual animals (open circles), and the mean is indicated by a horizontal line. The lower limit of detection is indicated by a dashed line.

cumming within 48 h (Fig. 1A). In contrast, mice given up to 10^8 CFU of YH102 or YH103 showed few adverse effects; some animals exhibited reduced movement for a few hours, but most recovered within 48 h (Fig. 1B and data not shown). The results demonstrate that the 50% lethal dose (LD_{50}) of both YH102 and YH103 is over two orders of magnitude greater than that for MC58.

The level of bacteremia was measured among animals 19 h after receiving MC58, YH102, or YH103; this time corresponds to the point of maximal bacteremia following infection with MC58. Following challenge with MC58, mice developed bacteremia in a dose-dependent fashion (Fig. 1C). In contrast, bacteremia was not detected in any animal ($n = 5$) receiving 10^8 CFU of YH102 or YH103 (lower limit of detection, 2×10^2 CFU/ml) at either 4 or 19 h after infection.

Immunization with YH102 or YH103 confers protection against live bacterial challenge. To test whether YH102 and YH103 can elicit protective immunity, groups of 30 animals were immunized i.p. on days 0 and 21 with 10^7 CFU of YH102 (MC58 Δ siaD Δ rfaF) or YH103 (MC58 Δ siaD Δ metH) in BHI-iron dextran. Control animals (30 mice in each group) were vaccinated with medium alone or with 25 μ g of purified PorA in Freund's incomplete adjuvant given subcutaneously. PorA is a surface-expressed protein that elicits bactericidal antibody responses against *N. meningitidis* (32). All animals remained healthy following immunization. On day 35, each group was divided to two sets of 15 animals and challenged with 10^6 or 10^7 CFU of MC58; the survival rates of animals after challenge are shown in Fig. 2.

Animals immunized with media alone became sick within 8 h of challenge. Within 24 h after challenge, the mice had lost weight and showed signs of distress, and by 72 h, a significant proportion had succumbed to infection. Vaccination with purified PorA conferred partial protection against high- and low-dose challenge ($P < 0.01$). However, animals that had been vaccinated with YH102 (MC58 Δ siaD Δ rfaF) or YH103 (MC58 Δ siaD Δ metH) appeared unaffected by challenge even with 10^7 CFU of MC58 (Fig. 2), which is between two to three orders of magnitude greater than the estimated LD_{50} for this strain. Similar results for protection from vaccination with YH102 and YH103 were obtained in three independent experiments.

To establish whether animals were also protected against bacteremia, the level of *N. meningitidis* organisms in the bloodstream was measured at 24 h after challenge with MC58; none of the animals vaccinated with YH102 or YH103 ($n = 4$) became bacteremic following challenge with the wild-type bacteria (Fig. 2).

Immunization with YH102 confers protection against heterologous strains. Whole-cell ELISAs were performed to establish whether antibodies raised in immunized animals recognized a range of meningococcal disease isolates. The strains were selected from a variety of serogroups and electrophoretic types (ETs) which span the diversity of pathogenic *N. meningitidis* strains (25). The results (Table 2) demonstrate that antibodies react with all strains tested at high titers. To determine if administration of the attenuated strains confers protection across multiple serogroups, challenge doses were established for strains 205900 (serogroup A, IV-I) and 90/18311 (serogroup C, ET-37) (data not shown). Groups of 10 animals were immunized with YH102 or YH103 on two occasions and then challenged with either the serogroup A or C strain at least 2 weeks after the final immunization. The survival rates of the animals were compared with those for animals immunized with BHI-iron dextran only and are shown in Fig. 3A and B. For both serogroups, animals immunized with the attenuated strain were partially protected, with more survivors among immunized than nonimmunized animals ($P < 0.05$ for challenge with 205900 and 90/18311). Similar results were obtained in replicate experiments. Following challenge with strain 205900, there were 8 survivors out of 10 in the immunized group, compared with 3 of 10 in the control group; for strain 90/18311, there were 8 and 4 survivors in the immunized and nonimmunized groups, respectively.

FIG. 2. Immunization with YH102 protects against live meningococcal challenge. Groups of 15 animals were vaccinated with YH102 given i.p. on days 0 and 21. Control animals (15 per group) were immunized with either recombinant PorA (given subcutaneously) or media alone (given i.p.). The survival of animals challenged with 10⁶ (A) or 10⁷ (B) CFU of MC58 on day 35 is shown. The levels of bacteremia at 24 h after challenge with 10⁷ CFU of MC58 are given in panel C. The results are for individual animals (open circles), and the mean is indicated by a horizontal line. Similar results were obtained following immunization with YH103 (not shown).

Protected animals develop bactericidal antibody responses. Next, we established whether animals vaccinated with the attenuated strain develop bactericidal antibodies, which mediate protection against serogroup C meningococcal infection (9). Sera were collected on day 28 from five animals that had been vaccinated with YH102 and from five unvaccinated control animals. The sera were tested for bactericidal activity against MC58 by using a baby rabbit complement source. Bactericidal activity was not detected in the sera from unvaccinated mice at a dilution of 1:4. In contrast, sera from vaccinated animals reproducibly killed over 50% of MC58 organisms at a dilution

TABLE 2. Whole-cell ELISA against a variety of *N. meningitidis* isolates

Strain	ET	Serogroup	ELISA titer ^a
MC58	ET-5	B	1,024
BZ169	ET-5	B	2,048
1000		B	512
NGP165	ET-37	B	512
90/18311	ET-37	C	256
A22		W135	256
205900	IV-1	A	2,048

^a The titer is expressed as the reciprocal of the dilution of sera from animals immunized with YH102 that gives a reading above the value obtained with preimmune sera.

of 1:1,024. There was detectable bactericidal activity against strains 205900 (SBA of 8) and 90/18311 (SBA of 64), though the titers are lower than those against the homologous strain.

YH102 and YH103 are defective for survival in the human WBA. Next we established whether the mutations in YH102 (MC58Δ*siaD*Δ*rfaF*) and YH103 (MC58Δ*siaD*Δ*methH*) attenuate *N. meningitidis* in the WBA to assess the strains' ability to survive within the human host. The WBA was performed with whole blood obtained from three individuals on at least two occasions; the level of killing of the wild-type strain was dependent on the donor used (Fig. 4A). However, YH102 and YH103 had a consistent defect for survival in the WBA compared to MC58, independent of donor. Neither YH102 nor YH103 was detectable after 30 min of incubation in the WBA using blood from any volunteer (Fig. 4B and C). In contrast, at the end of the incubation period, the wild-type strain was

FIG. 3. YH102 (MC58Δ*siaD*Δ*rfaF*) and YH103 (MC58Δ*siaD*Δ*methH*) elicit protection against challenge with serogroup A (panel A) and C (panel B) meningococcal challenge.

FIG. 4. YH102 (MC58 Δ siaD Δ rfaF) and YH103 (MC58 Δ siaD Δ metH) are defective for survival in the WBA. Bacteria (10^7) were inoculated into 1 ml of heparinized whole blood at time zero, and the number of viable organisms was measured at the indicated intervals and expressed as a proportion of the inoculum. Experiments were performed on two independent occasions. Results for the survival of MC58 are shown in panel A. Neither YH102 (B) nor YH103 (C) could be detected after 30 min of incubation, regardless of the donor.

detected at levels equivalent to or greater than the level of the inoculum.

DISCUSSION

In this report, we describe the selection of mutations to attenuate *N. meningitidis* from genes identified by signature-tagged mutagenesis (28). The mutations were transferred into strain MC58 as the available complete genome sequence of this bacterium (31) may prove valuable in future studies. Two mutations were introduced into each attenuated strain to reduce the possibility of genetic reversion to the wild-type. All mutations independently attenuate *N. meningitidis* in rodent models (21) and in the human WBA (data not shown). The mutations were chosen to block independent mechanisms required for full virulence, i.e., the polysaccharide capsule (*siaD*), LPS biogenesis (*rfaF*), and nutrient biosynthesis (*metH*). We found no apparent difference between the two attenuated strains, YH102 and YH103, in terms of their degree of attenuation in rodent models or in the WBA and of their ability to confer protection against live bacterial challenge.

Isolates of serogroup B *N. meningitidis* are characterized by the presence of the polysialic acid capsule at the surface of the bacterium. Expression of the capsule is an example of molecular mimicry that enables *N. meningitidis* to avoid recognition by the host immune system (10). Previous work demonstrated the importance of the capsule during meningococcal pathogenesis (12, 36), and capsular antigen inhibits deposition of complement on the bacterium (11), a major effector mechanism for killing pathogenic *Neisseria* (3). This may explain the rapid killing of both attenuated strains in the WBA. Furthermore, removal of the capsule from the surface of the bacterium may enhance the immune response to subcapsular antigens. Thus, both YH102 and YH103 contained an inactivated copy of *siaD*.

There are precedents of live attenuated vaccines expressing truncated LPS molecules. For example, the attenuated *S. enterica* serovar Typhi vaccine, Ty21a, has a defective *galE* allele (8). *rfaF* is required for the synthesis of part of the inner core of meningococcal LPS. LPS is a well-characterized virulence determinant for *N. meningitidis* that is involved in multiple steps in pathogenesis, including bloodstream dissemination (33). Recently, epitopes of inner-core meningococcal LPS, including the phosphoethanolamine on the beta chain, have been proposed as vaccine targets (26). We found that loss of this epitope, through deletion of *rfaF* (30), does not affect the ability of YH102 (MC58 Δ siaD Δ rfaF) to elicit protective immunity.

Mutations affecting genes encoding enzymes in metabolic pathways have been successfully introduced into live attenuated vaccines. Inactivated pathways include purine and aromatic amino acid biosynthesis (14, 18, 19, 29). However, our recent work highlighted the importance to *N. meningitidis* of methionine synthesis during systemic infection (28); four independent insertions were found in *metH* and *metF* which encode the enzymes for the final and penultimate steps in methionine synthesis, respectively. Our findings suggest that interruption of methionine biosynthesis could be effectively used to attenuate other bacteria.

Models of passive protection have been developed for meningococcal infection, but these can only assess the contribution of humoral immunity. The advantage of using adult mice is that it allows the active immunization of animals and assessment of protection against live bacterial challenge rather than having to rely on surrogate markers of immunity. However, *N. meningitidis* does not effectively colonize the rodent nasopharynx because of the specific binding of meningococcal surface molecules to human ligands (35); intranasal administration of the attenuated strains is unlikely to lead to colonization and subsequent immune responses in animals. Thus, we chose to inoculate the attenuated strains systemically rather than mucosally. When this route of infection was used, animals receiving either of the attenuated strains developed sterilizing immunity following challenge with the wild-type bacterium at a dose around two orders of magnitude higher than the LD₅₀ for unvaccinated animals. Furthermore, immunization with the attenuated strains led to the development of bactericidal antibodies and partial protection against challenge with heterologous meningococcal strains. The bacteria used for challenge were selected to be of different serotypes, subserotypes, and ETs from the vaccine strain. Therefore, this protection must be

mediated by meningococcal antigens other than PorA, such as TbfA and/or NspA (22, 37).

A significant advantage of using a murine model to assess protection is the wealth of resources for studying immune responses, including monoclonal antibodies for depleting specific subsets of effector cells and host strains lacking specific immunological markers. The large range of mice available with gene knockouts or depleting antibodies should allow dissection of the basic immunology underlying the protective responses.

Although we show here the protective efficacy of strains YH102 and YH103 and their marked attenuation in the human WBA, widespread implementation of live meningococcal vaccines may be problematic for several reasons. First, pathogenic *Neisseria* are competent for DNA uptake (4), providing a potential mechanism for genetic reversion; the likelihood of reversion could be reduced by deletion in the vaccine strain of gene(s) involved in transformation or recombination. Second, certain individuals (for example, those with complement deficiencies) (3) are at a much higher risk of developing meningococcal sepsis than the general population. These individuals may be vulnerable to developing meningococemia with attenuated strains. However, the attenuated strains could be used in murine models both to examine the basis of protection in more detail and to define the specific epitope(s) responsible for the development of sterilizing immunity. Furthermore, much remains to be understood of the nature of the immune response of individuals following exposure to live *N. meningitidis* serogroup B. Towards this goal, careful clinical trials could be undertaken in which healthy volunteers are given live attenuated *N. meningitidis* by mucosal administration and their immune responses intensively characterized.

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