Evaluation of Recombinant Lipidated P2086 Protein as a Vaccine Candidate for Group B Neisseria meningitidis in a Murine Nasal Challenge Model

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Neisseria meningitidis is a major causative agent of bacterial meningitis in human beings, especially among young children (≤2 years of age). Prevention of group B meningococcal disease presents a particularly difficult challenge in vaccine development, due to the inadequate immune response elicited against type B capsular polysaccharide. We have established an adult mouse intranasal challenge model for group B N. meningitidis to evaluate potential vaccine candidates through active immunization. Swiss Webster mice were inoculated intranasally with meningococci, and bacteria were recovered from the noses for at least 3 days postchallenge. Iron dextran was required in the bacterial inoculum to ensure sufficient meningococcal recovery from nasal tissue postchallenge. This model has been utilized to evaluate the potential of a recombinant lipidated group B meningococcal outer membrane protein P2086 (rLP2086) as a vaccine candidate. In this study, mice were immunized subcutaneously with purified rLP2086 formulated with or without an attenuated cholera toxin as an adjuvant. The mice were then challenged intranasally with N. meningitidis strain H355 or M982, and the colonization of nasal tissue was determined by quantitative culture 24 h postchallenge. We demonstrated that immunization with rLP2086 significantly reduced nasal colonization of mice challenged with the two different strains of group B N. meningitidis. Mice immunized with rLP2086 produced a strong systemic immunoglobulin G response, and the serum antibodies were cross-reactive with heterologous strains of group B N. meningitidis. The antibodies have functional activity against heterologous N. meningitidis strain, as demonstrated via bactericidal and infant rat protection assays. These results suggest that rLP2086 is a potential vaccine candidate for group B N. meningitidis.

Infections with Neisseria meningitidis represent a major health problem in both developed and developing countries. N. meningitidis serogroups A, B, C, W135, and Y account for approximately 95% of meningococcal disease worldwide; serogroups B and C cause the majority of meningococcal disease in developed countries, with 50 to 70% of those strains attributed to group B (1, 31). During the 1960s, polysaccharide vaccines were developed against groups A, C, W135, and Y; these have been shown to be immunogenic in human beings (2). Yet the immune response to these polysaccharide vaccines provides only limited protection for children <4 years of age, an age group that has significant disease burden, due to the nature of the immune response. To overcome this limitation, glycoconjugate vaccines are being developed against A, Y, and W135, and a group C conjugate has been introduced in a number of countries. However, the development of a capsular vaccine against group B is problematic, due to safety concerns and weak immunogenicity caused by the structural similarity between the capsular polysaccharide and human neural antigens (5, 35). As a result, other surface molecules, such as outer membrane proteins (OMPs) and lipooligosaccharides, are being evaluated as potential vaccines against group B N. meningitidis (18, 22, 37). One of the potential OMP vaccine candidates is the abundant and highly immunogenic PorA protein. However, the variable nature of this protein requires a multivalent vaccine composition to protect against a sufficient number of meningococcal serosubtypes found in clinical isolates (23, 32). The use of an antigen inducing cross-reactive bactericidal activity between serosubtypes would be preferable to a multivalent approach. Our search for an immunogenic component with broad cross-reactivity against multiple serosubtypes has led to the discovery of a lipidated protein designated LP2086 (6). LP2086 can be divided into two serologically distinct subfamilies (A and B) that induce bactericidal antibodies cross-reactive against strains within each respective P2086 family, regardless of the serosubtype antigens. Polyclonal antibody generated against recombinant LP2086 (rLP2086) killed multiple strains when tested in a bactericidal assay (6) and was protective in vivo in an infant rat passive-protection model (21). Recently, Masignani et al. also reported the vaccine potential of similar proteins (GNA 1870) encoded by the genome of N. meningitidis serogroup B strain, MC58, demonstrated by bactericidal and infant rat protection assays (16). The mature amino acid sequences of the two variants, P2086 derived from strain 8529 and NMB1870 derived from strain M58, are the same.

Meningococcal infection initiates from the adherence of the bacteria to human cells and results in the colonization of the organism on the nasopharyngeal mucosa (9). An effective meningococcal vaccine should provide protection against group B organisms either at the level of initial colonization, with bacterial invasion of the bloodstream, or through a combination of
both. Analysis of functional immune responses such as serum bactericidal activity, opsonophagocytosis activity, and passive immunization using in vivo bacteremia models enables us to characterize the induced responses of potential vaccine candidates. However, the development of meningococcal vaccines has been hampered by the lack of an animal model emulating the nasopharyngeal colonization and subsequent invasion into the bloodstream for use in evaluating potential vaccine candidates. Neonatal models have been used (24–26), but these can only be deployed for passive immunization. The lack of an adult animal colonization model has impeded analysis of potential vaccine candidates using active immunization. Recently, Yi et al. reported the development of an adult mouse model of meningococcal colonization; however, quantitative cultures were not reported in the paper (36).

In the present study, we developed an adult mouse intranasal (i.n.) challenge model for group B N. meningitidis and evaluated the vaccine potential of rLP2086 protein using active immunization and quantitative culture. Data presented here demonstrate that subcutaneous (s.c.) immunization with rLP2086 elicits antisera that are bactericidal and protect infant rats from meningococcal bacteremia. Subcutaneous immunization with rLP2086 also reduced nasal colonization in a newly developed adult mouse intranasal challenge model.

**MATERIALS AND METHODS**

**Animals.** Six-week-old, pathogen-free, female outbred Swiss Webster mice (Taconic Farms, Germantown, NY) and inbred BALB/c and C57BL/6 mice (Charles River Laboratories, Wilmington, ME) were used in the experiments. All animals were housed in a filtered HEPA Rack System under standard temperature, humidity, and lighting conditions prior to bacterial challenge. Food and water were available ad libitum.

**Bacterial strains and growth conditions.** Group B N. meningitidis strains H355 (B), H44/76 (B), M982 (B), 8529 (B), 870227 (B), 880049 (B), and 870446 (A) were obtained from the Centers for Disease Control and Prevention, Atlanta, GA. These isolates are representative of strains prevalent in western Europe and the Americas and contain representatives of both the A and B subfamilies of N. meningitidis. They were sacrificed and bled 3 h after challenge, and aliquots of blood were plated onto GCK plates and incubated overnight at 37°C with 5% CO2. The recovery of bacteria from the nasal tissue of these animals was compared on days 1, 2, and 3 post-nasal challenge.

Levels of bacteremia were determined by counting colonies on GCK plates after incubation at 37°C, 200 rpm, and 5% CO2. The ability of anti-rLP2086 antibodies to confer protection against N. meningitidis bacteremia was evaluated in infant rats challenged i.p. with 2×107 CFU of strain H44/76. They were sacrificed and bled 3 h after challenge, and aliquots of blood were plated onto GCK plates and incubated overnight at 37°C with 5% CO2. Levels of bacteremia were determined by counting colonies on GCK plates after incubation.

**Statistical analysis.** Statistical differences between groups were assessed by Student’s t test with an SAS statistical package (SAS Institute, Inc., Cary, NC). A P value of <0.05 was considered statistically significant.
RESULTS

Evaluation of susceptibility to meningococcal nasal colonization in adult mice. Three mouse strains were compared for susceptibility to intranasal colonization of N. meningitidis strain H355. As shown in Fig. 1, the best bacterial recovery was observed in Swiss Webster mice on the three consecutive days postchallenge. Approximately 3.5 to 5 log CFU were recovered from nasal tissue on days 1, 2, and 3 postchallenge. Approximately 3.5 to 4 log CFU of bacteria were recovered from nasal tissue of C57BL/6 mice on days 1 and 2 postchallenge. However, the recovery was decreased to about 1.5 log CFU on day 3. The recovery of bacteria from the nasal tissue of BALB/c mice was poor. Approximately 3 log CFU were recovered from nasal tissue on day 1 followed by minimal recovery on days 2 and 3. Based on these results, Swiss Webster mice were chosen for further model development. Figure 2 shows the results of i.n. challenge in Swiss Webster mice with three additional strains of group B N. meningitidis, i.e., strains 870227, M982, and CDC1521. The recovery of these three strains from noses was similar to the recovery following challenge with strain H355. Approximately 5 log CFU were recovered from nasal tissue on day 1 postchallenge, over 4 log CFU were recovered on day 2 and approximately 3 log CFU were recovered on day 3.

Both i.n. and i.p. iron supplements are necessary for significant enhancement of nasal colonization. Previous work using...
the meningococcal infant rat challenge model has shown that concurrent administration of iron with bacteria resulted in significantly enhanced levels of nasal colonization (26). Whether both i.n. and i.p. iron supplements are necessary was examined in this study. Female Swiss Webster mice, five mice per group, were each challenged i.n. with $1.7 \times 10^7$ CFU of group B \textit{N. meningitidis} H355 with or without 80 ug of iron dextran in the inoculum. Some groups of mice were also injected i.p. with iron dextran (2 mg/mouse) 4 h prior to and 24 h and 48 h after i.n. challenge. As shown in Fig. 3, significant recovery of bacteria was obtained only with mice given iron dextran both i.n. and i.p. Mice administered iron i.n. only showed good bacterial recovery on day 1 but very poor recovery on days 2 and 3. There was minimal recovery of bacteria on day 1 but no recovery on days 2 and 3 from mice administered iron i.p. only. Without any iron supplement, no bacteria were recovered on any of the days postchallenge.

**Reduction in nasopharyngeal colonization of \textit{N. meningitidis} cells after s.c. immunization with rLP2086.** The effect of s.c. immunization with rLP2086 was tested for the ability to protect against nasal colonization in the adult mouse nasal colonization model. Swiss Webster mice were vaccinated s.c. with 5 ug of purified rLP2086 protein administered with or without 10 ug CT-E29H or with CT-E29H alone. Two weeks after the last vaccination, mice were challenged i.n. with either $2.36 \times 10^7$ CFU of group B \textit{N. meningitidis} strain H355 or $1.98 \times 10^7$ CFU of M982. Nasal colonization was determined at 24 h postchallenge. As shown in Fig. 4A, mice immunized with rLP2086 in the presence or absence of CT-E29H had significantly lower colony counts of strain H355 in the nasal tissue than mice receiving CT-E29H alone or the naive mice control group ($P < 0.05$). Similarly, as shown in Fig. 4B, mice immunized with rLP2086 with or without CT-E29H also had significantly lower colony counts of strain M982 in the nasal tissue than mice receiving CT-E29H alone or the naive mice control group ($P < 0.05$). Animals immunized with rLP2086 plus CT-E29H had slightly lower CFU of either strain H355 (Fig. 4A) or M982 (Fig. 4B) than the rLP2086-immunized animals, although the difference was not significant.

**Serum antibody responses after s.c. immunization with rLP2086.** Swiss Webster mice immunized s.c. with 5 ug of rLP2086 protein with or without 10 ug of CT-E29H exhibited good rLP2086-specific IgG titers ($>10^6$) and low titers of IgA ($<100$). Adjuvant treatment with CT-E29H slightly increased the rLP2086-specific IgG antibody titers, even though the results were not statistically significant. However, addition of CT-E29H increased the levels of rLP2086-specific IgG2a and IgG2b antibodies approximately threefold (Table 1). In the mouse, IgG2a and IgG2b antibodies are the complement-fixing subclasses important for bactericidal activity. The immune sera also reacted with the cell surface of all eight group B meningococcal strains tested from both P2086 subfamilies (Table 2). It is noteworthy that bactericidal activity of the immune sera was observed against six of eight strains tested from both P2086 subfamilies and that adjuvancing with CT-E29H increased the bactericidal activity two- to fourfold against the five of eight strains tested (Table 3).

**Passive immunization with anti-rLP2086 antibodies reduced bacteremia in infant rats after challenge with meningococcal strain H44/76.** Sera from mice immunized s.c. with rLP2086 were passively transferred to infant rats to examine the effects on bacteremia postchallenge with \textit{N. meningitidis} group B strain H44/76. As shown in Fig. 5, the immune sera from mice immunized with rLP2086 with or without CT-E29H significantly reduced bacteremia in infant rats following i.p. challenge with meningococcal strain H44/76.

**DISCUSSION**

In this study, we developed an adult mouse intranasal challenge model for group B \textit{N. meningitidis} and evaluated rLP2086 protein as a vaccine candidate for the induction of
immune responses and protection against nasal colonization of *N. meningitidis* after challenge. An appropriate animal model is critical to evaluate the protective efficacy of a vaccine formulation. For meningococcal meningitis, the most commonly used active immunization-challenge model to examine the vaccine potential of an antigen has been the group B *N. meningitidis* challenge being administered by i.p. injection. This is an unnatural route of infection for meningococcal disease (3, 25, 33). Consequently, an i.n. challenge model, which mimics the natural route of infection, should provide a more meaningful way to evaluate vaccine candidates against group B meningococcus. We have previously successfully developed a nasal challenge of the infant rat as a model for evaluating meningococcal vaccines after passive immunization (26). However, the infant rat nasal

![Image](image_url)

**FIG. 4.** Immunization with rLP2086 reduced nasal colonization of group B *N. meningitidis* strains H355 or M982 following s.c. immunization and i.n. challenge in Swiss Webster mice. Six-week-old Swiss Webster mice, 10 mice per group, were vaccinated s.c. with 5 μg rLP2086 protein admixed with 10 μg CT-E29H at weeks 0 and 4. Groups of naïve mice and mice given CT-E29H alone were used as controls. Mice were challenged i.n. at week 6 with 2.36 × 10⁷ CFU *N. meningitidis* B H355 (A) or 1.98 × 10⁷ M982 (B) administered with iron dextran in the inoculum and i.p. (as described in the Fig. 3 legend). Noses were harvested, homogenized, and plated 24 h postchallenge. Results are expressed as log₁₀ CFU per nose ± SE. * values differ significantly from the naïve animal or CT-E29H control groups by Student’s *t* test (*P* < 0.05).

### TABLE 1. Vaccination with rLP2086 induces strong systemic immune responses in mice*

<table>
<thead>
<tr>
<th>Antigen (amt)</th>
<th>Adjuvant (amt)</th>
<th>IgG (mean log₁₀ ± SD)</th>
<th>IgG1</th>
<th>IgG2a</th>
<th>IgG2b</th>
<th>IgG3</th>
<th>IgA</th>
</tr>
</thead>
<tbody>
<tr>
<td>rLP2086 (5 μg)</td>
<td>None</td>
<td>5.94 ± 0.03</td>
<td>337,216</td>
<td>172,699</td>
<td>101,596</td>
<td>674</td>
<td>106</td>
</tr>
<tr>
<td>rLP2086 (5 μg)</td>
<td>CT-E29H (10 μg)</td>
<td>6.19 ± 0.31</td>
<td>404,055</td>
<td>453,147</td>
<td>327,294</td>
<td>1,195</td>
<td>83</td>
</tr>
</tbody>
</table>

* Swiss Webster mice (10/group) were vaccinated s.c. at weeks 0 and 4 and bled at week 6. The IgG antibody titers against rLP2086 were determined by ELISA of individual serum samples collected at week 6. The IgG subclass and IgA antibody titers against rLP2086 were determined by ELISA on pooled sera collected at week 6.
challenge model is limited to the evaluation of protective ef-

cacy of antisera that are passively administered. Therefore,

development of an adult animal colonization model is

crucial in evaluating vaccine efficacy following active immuni-

N. meningitidis is a strict human pathogen and does not

usually colonize the nasopharynx of a mouse. In this study, we

first compared the susceptibility of several outbred and inbred

strains of mice. The outbred Swiss Webster mouse strain was

identified as being more susceptible (Fig. 1); therefore, Swiss

Webster mice were used throughout these studies.

It is known that iron is essential for the growth and patho-
genesis of many pathogens, including N. meningitidis. While

iron is present in human tissues and blood in significant

amounts (~20 μM in blood), it is estimated that the concen-

tration of free iron in the blood is 10−18 M (10). The principal

agents responsible for iron sequestration in blood are trans-

ferrin (34) and heme in hemoglobin/haptoglobin complexes

(4). At mucosal surfaces, a frequent entry point for bacterial

pathogens, the glycoprotein lactoferrin sequesters iron (17).

Bacteria have developed several mechanisms for stripping iron

from these complexes; in the case of Neisseria meningitidis,

this harvesting of iron is done by transferrin binding and lactoferrin

binding proteins (28, 29). Previous investigators have used

transferrin, iron dextran, or mucin to satisfy the requirement

for exogenous iron and to ensure successful meningococcal

infection in animal models, particularly in i.p. infection models

(11–13, 24, 27). The results of our studies showed that the

presence of iron dextran significantly enhances the coloniza-
tion of nasal membranes of Swiss Webster mice and that both

i.p. administration of free iron in the blood is 10

18

has been well documented that serum bactericidal activity is a

major defense mechanism against meningococcal infection and

that protection against invasion by the bacteria correlates with

the presence of functional serum meningococcal antibodies (8,

9). Our results demonstrate an association between this in vitro

bactericidal activity of the immune sera and the reduction of

bacterial colonies in the nasal tissue from the immunized mice.

As seen from this study, s.c. immunization with rLP2086 protein

with or without adjuvant CT-E29H appears to offer a

promising approach for achieving protection from N. menin-

gitidis challenge (Fig. 4). In general for a protein subunit vaccine,

an adjuvant is often needed to enhance the antibody response,

and it was for this reason that CT-E29H was used in these

studies. CT-E29H is a mutant form of cholera toxin that has

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studies. CT-E29H is a mutant form of cholera toxin that has

that could be delivered. The challenge dose varied from experi-

ment to experiment (from 4.0 × 10^6 to 2.0 × 10^7 CFU)

during the development of the nasal colonization model. Once

we worked out the optimal conditions, we always used approx-

imately 2.0 × 10^7 CFU as a challenge dose for immunization-

challenge experiments. We have not detected bacteremia or

bacterial recovery from lungs after challenge in this model

system, even with a challenge dose as high as 2 × 10^9 CFU

(data not shown). This may be due to the low volume delivered

or to the inability of N. meningitidis to spread to the blood from

the nasopharynx of mice.

It is worth noting that the mouse i.n. colonization model and

the passive immune transfer model of bacteremia and meningi-

gitis are completely different and measure differing immune

mechanisms, opsonophagocytosis-bacteremia in one and clear-

ance-inhibition of mucosal colonization in the other. Active

immunization of adult Swiss Webster mice with rLP2086 pro-

tein showed significant reduction in nasopharyngeal coloniza-
tion after challenge with two different N. meningitidis B strains

from P2086 subfamily B in this newly developed model (Fig. 4).

After two immunizations, sera from these mice exhibited bac-
tericidal activity against several strains of N. meningitidis (Ta-

tle 3) and protected infant rats against bacteremia (Fig. 5). It

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REFERENCES


