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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 represents 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 OMP 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

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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 NVI (The Netherlands). The strain CDC1521 (A) was 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 LP2086, as indicated in parentheses following each strain. Strains used for the animal challenge experiments were passed twice through infant rats to enhance their colonization in animals (25) and then stored frozen at −70°C in GC medium (Difco, Detroit, MI) with Kellogg's supplement (GCK) containing 20% (vol/vol) glycerol (14). Additional passage of group B meningococcal strains in Swiss Webster mice did not improve the nasal colonization (data not shown). Prior to use in animal studies, the bacteria were inoculated onto Thayer Martin improved agar plates (Remel, Lenexa, KS) and incubated overnight at 37°C in an incubator containing 5% (vol/vol) CO_2. Colonies were removed from the agar plate by gentle washing with 5 ml of GCK, and an aliquot of this suspension was used to inoculate a culture flask containing 25 ml of GCK and grown to $A_{600} \cong 0.2$ after being inoculated. The bacterial suspension was incubated in an orbital shaker at 70 rpm and 37°C until the culture reached an optical density of $A_{600} \cong 0.8$ (3 to 4 h). This density was demonstrated to correspond to 1×10^9 to 3×10^9 CFU per ml. For the bactericidal assays, the bacteria were grown in a modified version of Frantz medium (glutamic acid, 1.3 g/liter; cysteine, 0.02 g/liter; sodium phosphate dibasic heptahydrate, 10 g/liter; potassium chloride, 0.09 g/liter; sodium chloride, 6.0 g/liter; ammonium chloride, 1.25 g/liter; 40 ml/liter yeast extract dialysate, and Kellogg's supplement) (7).

Purification of rLP2086. rLP2086 was expressed and purified as described previously (6). The P2086 gene is derived from a meningococcal group B strain, 8529, that belongs to the P2086 B subfamily. Purity was accessed by laser densitometry, following sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie blue staining. The purified protein exhibited >95% purity by these processes.

Intranasal challenge of adult mice. Six-week-old mice (5 to 15 per group) were anesthetized by injection with a mixture of ketamine (80 mg per kg of body weight) and xylazene (7 mg per kg of body weight) that maintains a state of anesthesia for 15 to 20 min. Mice were then challenged i.n. with 20 μl (10 μl/nostril) of the bacterial culture to which 80 μg of iron dextran (Sigma, St. Louis, MO) was added. All mice were also intraperitoneally (i.p.) administered with iron dextran (2 mg/mouse) 4 h prior to and 24 h and 48 h after i.n. challenge. At various times postchallenge, mice were sacrificed, and nasal tissues were homogenized and plated on Thayer Martin improved agar plates with 10-fold serial dilutions in saline. Bacterial colonies were enumerated after overnight incubation at 37°C in the presence of 5% CO_2. The recovery of bacteria from the nasal tissue of these animals was compared on days 1, 2, and 3 post-nasal bacterial challenge.

Immunization and bacterial challenge. Mice were immunized subcutaneously (s.c.) with rLP2086 (5 μg/mouse) admixed with or without CT-E29H (10 μg/mouse) (30) in a 0.2-ml volume at weeks 0 and 4. Control groups consisted of either unimmunized (naïve) mice or animals receiving CT-E29H (10 μg/mouse) alone. Sera were collected at weeks 0, 4, and 6 to determine the antibody responses and bactericidal activities. Two weeks after the last immunization, the animals were challenged i.n. with approximately 2×10^7 CFU of group B *N. meningitidis* as described above.

Determination of serum antibody levels to *N. meningitidis* whole cells or purified rLP2086. Antibody titers against rLP2086 were determined by enzyme-linked immunosorbent assay as previously described (6). Enzyme-linked immunosorbent assay titers against meningococcal whole cells were determined by using 96-well Costar plates coated with 100 μl of heat-killed (60°C for 1 h) *N. meningitidis* whole cells at an A_{600} of 0.1 in phosphate-buffered saline (PBS) (pH 7.2) and dried in a biosafety cabinet at room temperature. The remaining incubation times were 1 h at room temperature; the diluent for antibodies was PBS with 5% (wt/vol) nonfat milk. The coated plates were first blocked with 5% (wt/vol) nonfat milk in PBS and then incubated with serial dilutions of antisera. The bound primary antibodies were detected by biotinylated rabbit anti-mouse immunoglobulin G (IgG) antibodies (Brookwood Biomedical, Birmingham, AL), followed by streptavidin conjugated to horseradish peroxidase (Zymed Laboratories, Inc., San Francisco, CA). The color was developed for 30 min using ABTS [2,2′azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] containing H_2O_2 (Sigma) substrate solution. Absorbance was measured at 405 nm in a VERSAmax plate reader (Molecular Devices, Sunnyvale, CA). Titers are defined as the reciprocal of the serum dilution with an absorbance of 0.1.

SBA. A serum bactericidal assay (SBA) was performed as previously described (19) with human serum from individual donors as the complement source. Briefly, assay components consisted of 25 μl of PBS with calcium and magnesium at pH 7.4 (PCM buffer), 5 μl of heat-inactivated (56°C for 30 min) serially diluted (twofold dilution) test serum, 10 μl of human complement, and 10 μl of PCM buffer containing approximately 1×10^3 to 3×10^3 viable *N. meningitidis* organisms. The complement source used had no bactericidal activity against the target bacterial strain. Following a 30-min incubation of the assay mixture at 37°C, 200 μl of Alamar blue dye (Trek Diagnostic Systems, Westlake, OH) at a 1:20 dilution in modified Frantz growth medium containing 0.7% low-melting-point agarose was added to each well. The assay plate was then incubated at 37°C overnight in a Cytofluor 4000 fluorescent plate reader (Perceptive Biosystems, Framingham, MA), which reads the fluorescent signal every 30 min. Wells containing known numbers of target cells without test serum were included on each assay plate and used to generate a standard curve. A serum with known bactericidal titer was used as a positive serum control. In this study, the SBA was performed on pooled serum specimens from weeks 0 and 6. Titers were reported as the reciprocal of the greatest dilution that yielded ≥50% bacterial killing compared to assay controls. Specimens that demonstrated <50% killing at the lowest serum dilution tested (the lowest dilution tested for serum samples was 1:25) were reported as having a SBA titer of <25.

Infant rat protection assay. The ability of anti-rLP2086 antibodies to confer protection against *N. meningitidis* bacteremia was evaluated in infant rats challenged i.p. as previously described (20). Briefly, 3- to 4-day-old pups from litters of outbred Sprague-Dawley rats (Charles River Laboratories, Wilmington, ME) were randomly redistributed to the nursing mothers. Groups of 10 infant rats were injected i.p. with 1:10 dilutions of mouse anti-rLP2086 serum 18 to 24 h prior to challenge. They were then challenged i.p. with 2.1×10^5 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% CO_2. 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.

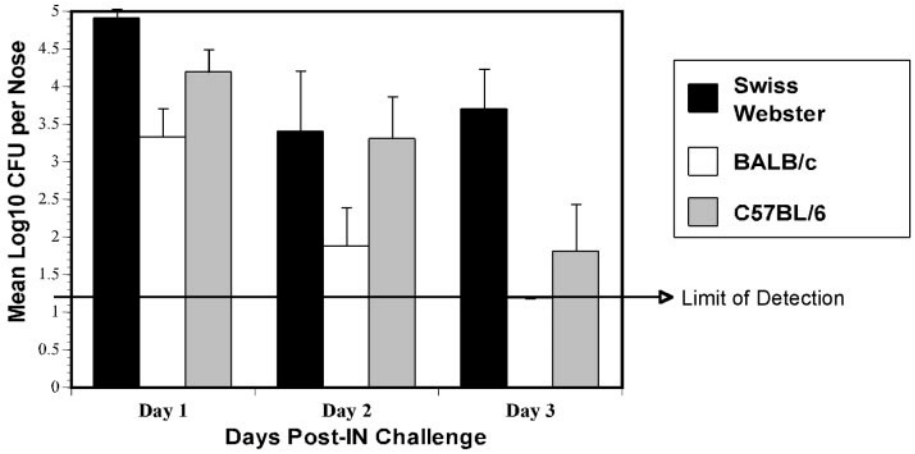

FIG. 1. Evaluation of group B *N. meningitidis* i.n. challenge in three different mouse strains. Swiss Webster, BALB/c, and C57BL/6 mice (6 weeks old, 15 mice per group) were i.n. challenged with 20 μl (10 μl/nostril) of group B *N. meningitidis* H355 (4×10^6 CFU) containing 80 μg of iron dextran. All mice were also injected i.p. with 2 mg of iron dextran 4 h prior to and 24 and 48 h after intranasal challenge. Five mice from each group were sacrificed, and nasal tissues were homogenized and plated on days 1, 2, and 3 postchallenge. Bacterial recovery from the nose is expressed as $\log_{10}$ CFU ± standard error (SE).

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

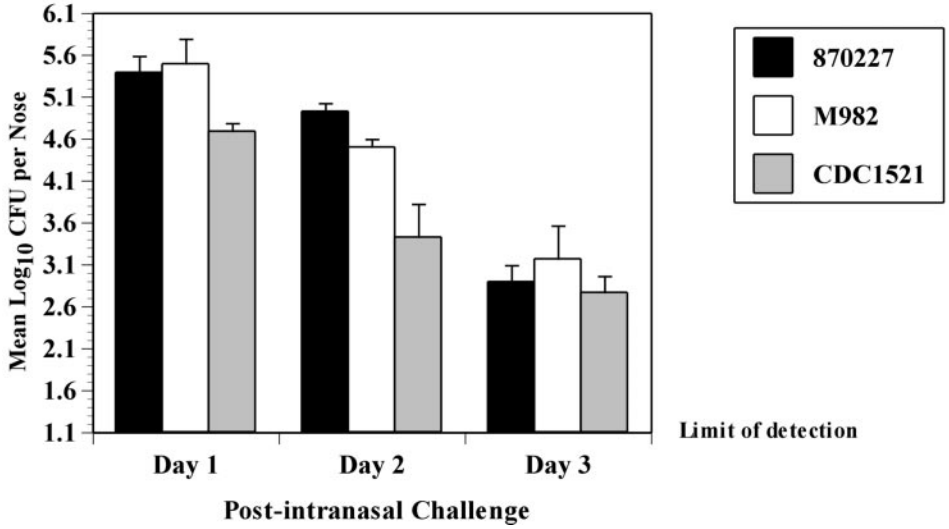

FIG. 2. Comparison of intranasal challenge with three different strains of group B *N. meningitidis* in Swiss Webster mice. Six-week-old Swiss Webster mice, 15 mice per group, were i.n. challenged with group B *N. meningitidis* strains 870227, M982, and CDC1521, at 4×10^6 CFU each in 20 μl containing 80 μg of iron dextran. Five mice from each group were sacrificed, and nasal tissues were homogenized and plated on days 1, 2, and 3 postchallenge. Bacterial recovery from the nose is expressed as $\log_{10}$ CFU per nose ± SE.

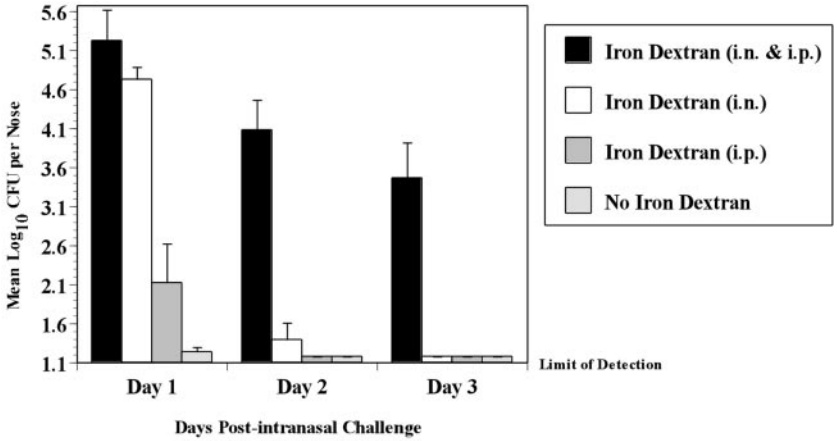

FIG. 3. Iron dextran is required for nasal colonization of group B *N. meningitidis* in Swiss Webster mice. Six-week-old Swiss Webster mice, 15 mice per group, were used in this experiment. One group of mice was injected i.p. with 2 mg of iron dextran 4 h prior to and 24 h after challenge. This group of mice was challenged i.n. with 1.7×10^7 CFU of group B *N. meningitidis* H355 with 80 μg of iron dextran. Another group of mice (without i.p. supplementation of iron dextran) were challenged i.n. with 1.7×10^7 CFU of group B *N. meningitidis* H355 with 80 μg of iron dextran. The third group of mice received iron dextran i.p. as described for the first group of mice and was challenged with the same amount of bacteria in the absence of iron dextran. The fourth group of mice received the bacterial challenge without any supplement of iron dextran i.p. or i.n. Nasal tissues were harvested, homogenized, and plated from five mice of each group 1, 2, and 3 days postchallenge. Results are expressed as $\log_{10}$ CFU per nose ± SE.

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 1.7×10^7 CFU of group B *N. meningitidis* H355 with or without 80 μg 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 *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 μg of purified rLP2086 protein administered with or without 10 μg CT-E29H or with CT-E29H alone. Two weeks after the last vaccination, mice were challenged i.n. with either 2.36×10^7 CFU of group B *N. meningitidis* strain H355 or 1.98×10^7 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 naïve mice control groups ($P < 0.05$). 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 naïve 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 μg of rLP2086 protein with or without 10 μg of CT-E29H exhibited good rLP2086-specific serum 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 adjuvanting 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 *N. meningitidis* group B strain H44/76. As shown in Fig. 5, the immune sera from mice immunized with rP2086 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 *N. meningitidis* and evaluated rLP2086 protein as a vaccine candidate for the induction of

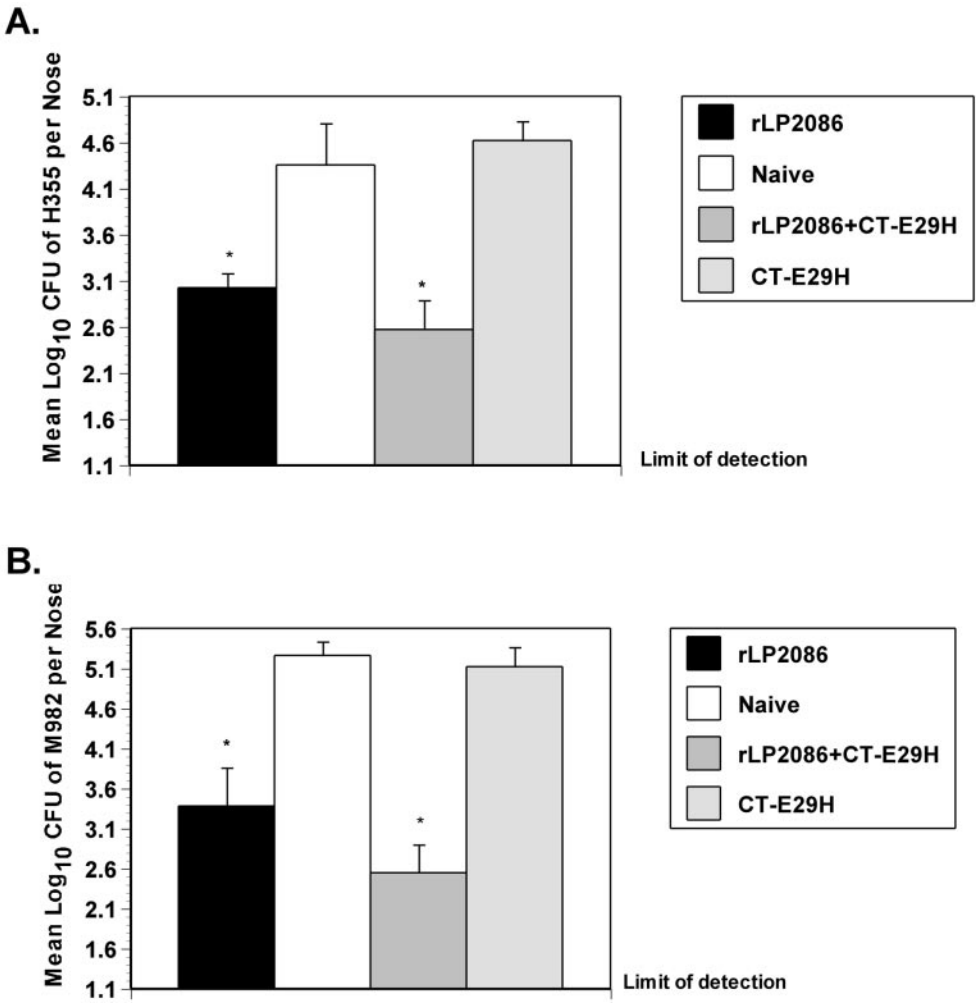

FIG. 4. Immunization with rLP2086 reduced nasal colonization of group B *N. meningitidis* strains H355 or M982 following s.c. immunization and i.n. challenge in Swiss Webster mice. Six-week-old Swiss Webster mice, 10 mice per group, were vaccinated s.c. with 5 μg rLP2086 protein admixed with 10 μg CT-E29H 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 2.36×10^7 CFU *N. meningitidis* B H355 (A) or 1.98×10^7 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 $\log_{10}$ CFU per nose ± SE. *, values differ significantly from the naïve animal or CT-E29H control groups by Student's *t* test ($P < 0.05$).

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

TABLE 1. Vaccination with rLP2086 induces strong systemic immune responses in mice[a]

Antigen (amt)	Adjuvant (amt)	Titer					
		IgG (mean $\log_{10}$ ± SD)	IgG1	IgG2a	IgG2b	IgG3	IgA
rLP2086 (5 μg)	None	5.94 ± 0.03	337,216	172,699	101,596	674	106
rLP2086 (5 μg)	CT-E29H (10 μg)	6.19 ± 0.31	404,055	453,147	327,294	1,195	83

[a] 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.

TABLE 2. Serum IgG antibodies elicited by rLP2086 cross-reacted to multiple meningococcal strains[a]

Antigen (amt)	Adjuvant (amt)	Test strain[b]							
		8529	H44/76	H355	880049	M982	870227	870446	CDC1521
rLP2086 (5 μg)	None	5.03 ± 0.22	4.72 ± 0.15	4.47 ± 0.17	4.06 ± 0.12	3.72 ± 0.14	4.82 ± 0.18	4.16 ± 0.12	3.59 ± 0.13
rLP2086 (5 μg)	CT-E29H (10 μg)	5.20 ± 0.26	4.96 ± 0.23	4.62 ± 0.27	3.78 ± 0.37	3.86 ± 0.22	4.92 ± 0.28	3.76 ± 0.54	3.39 ± 0.21

[a] Swiss Webster mice (10/group) were vaccinated s.c. at weeks 0 and 4 and bled at week 6. The geometric mean serum IgG titers against whole cells of various meningococcal strains were determined by ELISA of individual serum samples collected at week 6 and reported as $\log_{10}$ ELISA titers ± standard deviation.
[b] All strains belong to P2086 subfamily B, except strains 870446 and CDC1521.

challenge model is limited to the evaluation of protective efficacy of antisera that are passively administered. Therefore, the development of an adult animal colonization model is crucial in evaluating vaccine efficacy following active immunization.

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 pathogenesis 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 concentration of free iron in the blood is 10^{-18} M (10). The principal agents responsible for iron sequestration in blood are transferrin (34) and heme in hemagloblin/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 colonization of nasal membranes of Swiss Webster mice and that both i.p. and i.n. administration of iron was required for nasal colonization of group B *N. meningitidis* in adult mice (Fig. 3).

In addition, we chose a low inoculum volume (10 μl per nare) to ensure that the initial colonization was restricted to the nasopharynx. Higher inoculum volumes (20 to 50 μl per nare) tend to spread into the trachea and the lungs. Due to this volume restriction, we were limited in the number of bacteria that could be delivered. The challenge dose varied from experiment 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 approximately 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 2×10^8 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 nasopharynxes of mice.

It is worth noting that the mouse i.n. colonization model and the passive immune transfer model of bacteremia and meningitis are completely different and measure differing immune mechanisms, opsonophagocytosis-bacteremia in one and clearance-inhibition of mucosal colonization in the other. Active immunization of adult Swiss Webster mice with rLP2086 protein showed significant reduction in nasopharyngeal colonization 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 bactericidal activity against several strains of *N. meningitidis* (Table 3) and protected infant rats against bacteremia (Fig. 5). It 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. meningitidis* 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

TABLE 3. Serum bactericidal activity against *N. meningtitidis* in mice vaccinated with rLP2086 ± CT-E29H[a]

Antigen (amt)	Adjuvant (amt)	Bactericidal titers against test strain[b]							
		8529	H44/76	H355	880049	M982	870227	870446	CDC1521
rLP2086 (5 μg)	None	400	400	100	<25	>800	<25	50	<25
rLP2086 (5 μg)	CT-E29H (10 μg)	>800	>800	400	50	>800	<25	200	<25

[a] Swiss Webster mice (10/group) were vaccinated s.c. at weeks 0 and 4 and bled at week 6. Serum bactericidal activity of various group B meningococcal strains was assessed with week 6 pooled sera. Human complement (lot# UR4-97) was used in the assay. The bactericidal titers are expressed as the reciprocal of the greatest serum dilution that yielded ≥50% of bacterial killing, compared to assay controls. The bactericidal titers of all the prebleeds were <25.
[b] All strains belong to P2086 subfamily B, except strains 870446 and CDC1521.

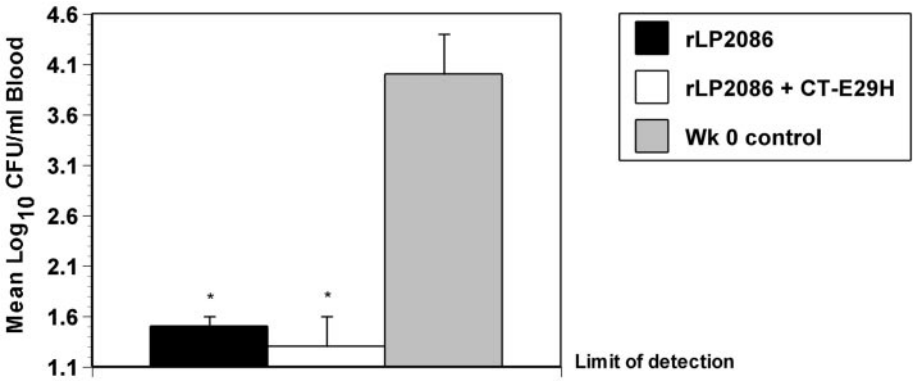

FIG. 5. Passive immunization with anti-rLP2086 antibodies reduced bacteremia in infant rats after challenge with meningococcal strain H44/76. Three- to 4-day-old Sprague-Dawley rats, 10 rats per group, were injected i.p. with 1:10 dilutions of the week 6 mouse sera from mice immunized s.c. with rLP2086 (rLP2086) or rLP2086 given as an adjuvant with CT-E29H (rLP2086 plus CT-E29H). The prebleed (week 0 control) was used as a control. Eighteen to 24 h later, rats were then challenged i.p. with 2.1×10^5 CFU of group B meningococcal strain H44/76. Rats were sacrificed and bled 3 h postchallenge. Aliquots of blood were plated onto GCK plates and incubated overnight at 37°C with 5% CO_2. Levels of bacteremia were determined by counting colonies on GCK plates after incubation. *, values differ significantly from the week 0 control group by Student's *t* test ($P < 0.05$).

reduced enzymatic activity and <1% of the cellular toxicity of native cholera toxin but remains fully active as an adjuvant, which suggests promise for use in humans (30). CT-E29H appears to be a promising adjuvant choice for rLP2086 in this study, as CT-E29H increased rLP2086-specific Th1 immune response, as evident by the increasing IgG2a and IgG2b antibody titers (Table 1) and bactericidal activities (Table 3). It also appears that CT-E29H enhanced protection against bacteremia in infant rats and against nasal colonization in Swiss Webster mice after challenge. Since CT-E29H is a detoxified cholera toxin, further animal toxicology testing must be done before CT-E29H can be delivered to people. While there is some concern about administering genetically detoxified enterotoxins as mucosal adjuvants (15), these concerns may not apply to parenteral administration of these molecules. To determine if parallel responses can be elicited in people, delivery of rLP2086 with CT-E29H should be tested in a clinical trial.

In summary, we have developed an adult mouse intranasal challenge model for group B *N. meningitidis* and have used it to evaluate the vaccine potential of our recombinant lipoprotein, rLP2086. We showed that s.c. immunization of Swiss Webster mice with purified rLP2086 protein given as an adjuvant with or without CT-E29H induced rLP2086-specific serum IgG antibodies that recognized surface-exposed P2086 epitopes on various strains of group B *N. meningitidis* from the two LP2086 subfamilies. The serum antibodies had bactericidal activity directed against multiple strains of group B *N. meningitidis* from the two LP2086 subfamilies; passive immunization with these sera reduced bacteremia in infant rats following *N. meningitidis* challenge. Subcutaneous immunization with rLP2086 given as an adjuvant with or without CT-E29H reduced nasal colonization of two strains of group B *N. meningitidis* using our newly developed adult mouse intranasal challenge model.

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