A Novel Live Vector Group A Streptococcal emm Type 9 Vaccine Delivered Intranasally Protects Mice against Challenge Infection with emm Type 9 Group A Streptococci

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The availability of a protective vaccine against Streptococcus pyogenes (group A Streptococcus [GAS]) is a priority for public health worldwide. Here, we have generated six live vaccine strains, each engineered to express an N-terminal M protein peptide from one of six of the most prevalent emm types of GAS (M1, M2, M4, M9, M12, and M28). The vaccine strains are based on a food-grade Lactococcus lactis strain and do not bear any antibiotic resistance. Mice immunized with the vaccine strain expressing the M9 peptide (termed here the L. lactis M9 strain) showed high titters of serum antibodies when delivered intranasally. Mice immunized with the L. lactis M9 strain were protected against infection after intranasal challenge with type 9 streptococci. Several parameters of disease, such as weight loss, body temperature, colony counts in mouth washes, and lung histology, were significantly improved in immunized mice compared to naive control mice. Our results indicate that intranasal delivery of the L. lactis M9 strain live bacterial vaccine induced GAS-specific IgG titters, prevented pharyngeal colonization of GAS, and protected mice from disease upon challenge. The design of this vaccine prototype may provide a lower cost alternative to vaccines comprised of purified recombinant proteins.

Streptococcus pyogenes (group A Streptococcus [GAS]) is an exclusively human pathogen that can cause a variety of diseases in immunocompetent individuals, ranging from uncomplicated superficial infections, such as tonsillopharyngitis, to severe life-threatening infections, including necrotizing fasciitis and toxic shock syndrome (1). Moreover, GAS infection may result in autoimmune disorders, such as rheumatic fever and rheumatic heart disease (2). Globally, more than 18 million people are estimated to suffer from a severe disease caused by GAS (3). In a study of Chilean patients diagnosed with tonsillopharyngitis, GAS was detected in 37% of cases (4). A recent (December 2013) report from the Public Health Institute of Chile (5) indicated that invasive GAS disease has increased by approximately 30% from 2009 to 2013. A study made in the United States estimated an economic cost of $224 to $539 million dollars per year due to tonsillopharyngitis (6, 7). Therefore, infection with GAS remains a significant public health burden worldwide.

GAS colonizes tonsils, skin, and oral and nasal mucosa and is able to invade deeper tissues. GAS virulence depends on a variety of secreted and surface proteins that promote host invasion as well as evasion of the immune response (8). Because GAS is an extracellular pathogen, a major virulence mechanism is the ability to resist phagocytosis, whereas the major defensive mechanisms of the host are both innate and adaptive immune responses. The immediate innate immune response to GAS involves resident macrophages (9) and polymorphonuclear leukocytes (PMNs) and natural killer cells recruited to the site of infection (10). Adaptive immunity against GAS, consisting of high titters of opsonic antibodies, has been associated with decreased rates of symptomatic infection (11). Opsonic antibodies against the N-terminal domain of M protein are essential for effective clearance of this pathogen (12).

M proteins are cell wall-anchored proteins that have an important role in resistance to phagocytosis (13). The N-terminal domain of the M protein is surface exposed and exhibits extensive variability in its sequence. According to the Streptococcus pyogenes emm Sequence Database available at the Centers for Disease Control and Prevention website (http://www2a.cdc.gov/ncidod/biotech/strepblast.asp), there are more than 200 different M proteins based on this variable region. M protein is encoded by the emm gene. The N-terminal domain of M proteins elicits antibodies with high bactericidal (protective) activity (14) and is considered a viable candidate vaccine antigen. We recently conducted a study of the molecular epidemiology of GAS infections in Chile and determined the emm type distribution (15). This knowledge was applied to select the most common emm types to include them in the design of this new vaccine. M protein peptides derived from the emm types 1, 2, 4, 9, 12, and 28 were individually expressed in the L. lactis strain and do not bear any antibiotic resistance. M9 strain) showed high titers of serum antibodies when delivered intranasally. Mice immunized with the L. lactis M9 strain were protected against infection after intranasal challenge with type 9 streptococci.
PCR amplification of emm recombinant live vaccines. GAS isolates of each emm type were used for PCR amplification of emm gene fragments. In order to allow the protein to be exported, every emm gene fragment was amplified with its corresponding signal peptide (SP) sequence using primers bearing restriction enzyme (RE) sites. In order to achieve surface display of the hybrid protein, every peptide included the C-terminal membrane anchoring domain (MAD) of M6 protein (148 amino acids, including the LPXTGE motif). Each emm gene fragment was digested with RE and ligated to the MAD fragment. The resulting fusion fragment was digested with RE and introduced into the linearized vector pNZ8149. Each construct was established in the food-grade Lactococcus lactis strain NZ3900 through electroporation. Six different recombinant lactococcal strains were obtained: the M1, M2, M4, M9, M12, and M28 strains. The recombinant M9 strain was used to immunize mice in order to achieve surface display of the hybrid protein, every peptide included the C-terminal membrane anchoring domain (MAD) of M6 protein (148 amino acids, including the LPXTGE motif). Each emm gene fragment was digested with RE and ligated to the MAD fragment. The resulting fusion fragment was digested with RE and introduced into the linearized vector pNZ8149. Each construct was established in the food-grade Lactococcus lactis strain NZ3900 through electroporation. Six different recombinant lactococcal strains were obtained: the M1, M2, M4, M9, M12, and M28 strains. The recombinant M9 strain was used to immunize mice in which the effectiveness of immunization was evaluated after challenge with emm type 9 GAS.

Materials and Methods

Strains, media, and culture conditions. The NICE genetic system was used for the constructions (NICE System, MoBiTech, Germany). It possesses an antibiotic-free selection system based on the auxotrophy for lactose utilization. It consists of the vaccine vector L. lactis NZ3900, a food-grade strain commonly used in the dairy industry, which lacks the lacF gene, necessary for lactose utilization, and the expression plasmid pNZ8149, which bears the lacF gene and possesses a nisin-inducible promoter. The wild-type (WT) strain, NZ2900, was grown in M17 agar (Sigma) supplemented with glucose (5 g/liter). All of the transformed strains were grown in M17 agar supplemented with lactose (5 g/liter) and bromocresol purple (0.04 g/liter) (Sigma). For the preparation of the infective inoculum, an erythromycin-resistant emm type 9 GAS clinical isolate (strain no. 75) that had been previously reported (15) was grown in Todd-Hewitt broth overnight at 37°C. Mouth washes were plated in Trypticase soy agar supplemented with 5% sheep’s blood and erythromycin (2 µg/ml).

Vaccine construction. The first 30 to 50 amino acids of the hypervariable domain of M proteins have been shown to evoke antibodies with the greatest protective activity and have minimal risk of cross-reacting with human tissues (17–19). Peptides of 30 to 50 amino acids long were selected using the bioinformatic tool BLAST; those sequences resembling human proteins and likely to elicit autoreactive antibodies were excluded. The sequence of the hypervariable domain used for each M protein is shown in Table 1. GAS isolates of emm types 1, 2, 4, 9, 12, and 28 were used for PCR amplification of the emm gene fragment: the forward and reverse primers have the restriction enzyme (RE) sites for Ncol and Clal, respectively. In order to achieve surface display of the hybrid protein, the M peptide was fused to 148 amino acids from the C-terminal membrane used to stimulate mucosal and systemic immune responses against a pathogen that enters a mammalian host at a specific site (e.g., oral) (16). Based on this rationale, a vaccine was designed consisting of a mixture of the six different recombinant bacterial strains, each one expressing an individual M protein (Fig. 1). Here we show that immunization of BALB/c mice with Lactococcus lactis expressing M9 peptide (here termed the L. lactis M9 strain) confers protection against subsequent challenge with type 9 GAS. Immunoglobulin titers in serum from immunized mice and parameters of disease, such as weight loss, body temperature, colony counts in mouth washes, and lung histology, were measured to evaluate the effectiveness of the vaccine in eliciting protective immunity.

Table 1. Sequence of the hypervariable domain included in each vaccine

| M protein | Sequence/final position no. (in original GAS protein) | Hypervariable domain length (aa) |
|-----------|-----------------------------------------------------|---------------------------------|
| M1        | Asn-Gly-Asp-Gly-Asn-Pro-Arg-Glu-Val-Ile-Glu-Asp-Leu-Ala-Ala-Asn-Pro-Arg-Ala-Ile-Arg-Leu-Arg-His-Glu-Asn-Lys-Asp-Leu-Lys-Ala-Arg-Leu-Glu-Asn-Ala-Met/81 | 40 |
| M2        | Asn-Ser-Lys-Asn-Pro-Val-Pro-Val-Lys-Glu-Ala-Lys-Leu-Ser-Glu-Ala-Glu-Leu-His-Asp-Lys-Ile-Lys/65 | 24 |
| M4        | Ala-Glu-Ile-Lys-Lys-Pro-Gln-Ala-Asp-Ser-Ala-Trp-Asn-Trp-Pro-Glu-Lys-Tyr-Asn-Ala-Leu-Leu-Lys-Glu-Asn-Glu-Leu-Val-Glu-Arg-Glu-Lys-Tyr-Leu-Ser-Tyr-Val-Ala-Asp-Asp-Lys-Glu-Lys-Asp-Pro-Gln-Tyr-Arg-Ala/91 | 50 |
| M9        | Gly-Glu-Val-Lys-Lys-Ala-Glu-Ala-Ala-Leu-Val-Pro-Lys-Thr-Glu-Tyr-Asp-Leu-Leu-Tyr-Asp-Leu-Asp-Leu-Asp-Leu-Asp-Leu-Arg-Glu-Glu-Glu-Arg-Gln/87 | 46 |
| M12       | Asp-His-Ser-Asp-Ala-Val-Ala-Lys-Glu-Ala-Glu-Leu-Ser-Glu-Leu-Arg-Ile-Glu-Glu-Leu-Glu-Glu-Arg-Gln/87 | 49 |
| M28       | Ala-Glu-Ser-Pro-Arg-Ser-Thr-Glu-Thr-Ser-Ala-Asn-Gly-Ala-Asp-Leu-Ala-Asp-Ala-Tyr-Asn-Thr-Leu-Leu-Thr-His-Glu-Lys-Leu-Arg-Asp-Glu-Tyr-Tyr-Thr-Leu-Ile-Asp-Ala-Lys-Glu-Glu-Glu-Pro-Arg-Tyr-Lys-Ala/83 | 50 |
anchoring domain (MAD) of M6 protein. Forward and reverse primers for the MAD fragment have the RE sites for ClaI and XbaI, respectively. The resulting fusion fragment was digested with NcoI and XbaI and ligated to the linearized vector pNZ8149. In order to verify the correct ligation, PCR was performed with primers that hybridize in the plasmid outside the insert (Ins-F and Ins-R) and amplify a fragment of ~500 bp. Plasmids were introduced into L. lactis strain NZ3900 through electroporation. Transformed clones were selected in M17-lactose-hromocresol purple. PCR of yellow colonies was performed to confirm that the colonies contained the plasmid. Six vaccine strains were obtained: the L. lactis M1, M2, M, M9, M12, and M28 strains. The L. lactis wild-type (WT) control strain was obtained after transformation of L. lactis strain NZ3900 with the empty plasmid pNZ8149.

Vaccine production: expression of recombinant proteins in L. lactis. For protein expression, recombinant L. lactis strains were grown overnight at 30°C in M17-lactose medium. This culture was used to inoculate 10 ml of fresh medium and grown under the same conditions until an optical density at 600 nm (OD600) of 1 was reached. At this moment, the culture was divided, and nisin was added at 0, 5, 10, and 20 ng/ml. Cultures were incubated for 3 h at 30°C. Cultures were centrifuged at 5,000 × g for 5 min. Pellets were resuspended in phosphate-buffered saline (PBS) with protease inhibitor (Cell Signaling) and lysosome (1 mg/ml) and sonicated on ice for 90 s, and the resulting pellets were dissolved in 50 mM NaOH and analyzed using 12.5% polyacrylamide gel electrophoresis with SDS (SDS-PAGE). Supernatants were filtered with 0.2-µm filters, and their proteins were precipitated with 16% trichloroacetic acid (TCA) and centrifuged at 10,000 × g for 10 min, and the resulting pellets were dissolved in 50 mM NaOH and analyzed by SDS-PAGE. Polyacrylamide gels were stained overnight with Coomassie blue (2/1000).

Immunization and challenge of mice. All animal experiments were performed according to protocols approved by the Bioethics and Biosafety Committee of the Pontifícia Universidade Católica do Brasil. Two independent experiments of immunization and challenge were performed to test the M9 vaccine. For L. lactis M9 strain vaccine dose preparation, 1 ml of the freshly induced culture of the M9 strain (with 20 ng/ml nisin) was centrifuged and resuspended in 50 µl of PBS, which was used to immunize one mouse. For L. lactis WT control vaccine dose preparation, 1 ml of freshly induced culture of the L. lactis WT strain (with 20 ng/ml nisin) was centrifuged and resuspended in 50 µl of PBS, which was used to immunize one mouse. Groups of 3 to 4 female BALB/c mice (6 to 8 weeks-old) were intranasally immunized with 10^6 CFU of the L. lactis M9 or L. lactis WT strain or unimmunized (received only sterile PBS). The control group of mice immunized with the L. lactis WT strain was included in order to evaluate the potential protective effect of the bacterial vector itself. Booster immunizations of the same dose were given 14 and 28 days following the initial immunization. Blood samples were obtained from all animals at days 0, 14, 28, and 42. Blood was incubated for 1 h at 37°C and centrifuged for 5 min at 5,000 rpm. Serum was transferred to a clean tube and stored at 4°C. The infective dose was previously tested in BALB/c mice infected with different bacterial doses (2 × 10^3, 2 × 10^4, and 2 × 10^5 CFU). Mice infected with 2 × 10^4 CFU underwent around 20% body weight loss, had a temperature decrease of around 2°C, and exhibited an appearance and attitude typical of diseased mice. Fourteen days after the second boost, intranasal challenge was performed with 2 × 10^8 CFU of emm type 9 GAS in a total volume of 50 µl of PBS. After the challenge, body temperature and body weight were determined each day. Body temperature was measured with an infrared digital thermometer. Mouth washes were performed for three consecutive days after challenge. Fifty microliters of PBS was pipetted into the mouse mouth up and down 5 times and finally plated on blood agar supplemented with 2 µg/ml erythromycin. Mice were anesthetized with isoflurane prior to mouth washes. Six days after challenge, mice were sacrificed and lungs were extracted.

Immunogenicity evaluation. M9-specific IgG in serum was determined by enzyme-linked immunosorbent assay (ELISA). Synthetic peptide (5 µg/ml) copyng the N-terminal 46 amino acids of M9 (Peptide 2.0, Inc., Chantilly, VA) was bound to flat-bottom 96-well microtiter plates in 0.1 M sodium carbonate-bicarbonate buffer, pH 9.6 (100 µl/well), overnight at room temperature. Excess peptide was removed, and wells were washed five times with PBS–0.05% Tween 20 (PBS–T). Wells were blocked with PBS–2% bovine serum albumin (BSA) for 1 h at 37°C and then washed 3 times with PBS–T. Mouse sera were serially diluted 1:2 with PBS–T–0.5% BSA starting from 1/100, and 100 µl of each dilution was added to the wells and incubated for 1 h at 37°C. The wells were washed 3 times with PBS–T, 100 µl of enzyme conjugate (horseradish peroxidase conjugated with anti-mouse IgG) was added, and the mixture was incubated for 1 h at 37°C. Wells were washed 3 times with PBS–T, 100 µl of substrate solution (tetramethylbenzidine; Thermo Scientific) was added, and the mixture was incubated for 6 min in the dark at room temperature. The reaction was stopped with 100 µl of H2SO4 (2 M), and the plates were read at 450 nm. The antibody titer was defined as the reciprocal of the highest dilution of serum that yielded an absorbance of ≥0.1 at 450 nm.

Histopathology. Lungs were removed 6 days after challenge and fixed in formalin. Fixed tissue was embedded in paraffin, sectioned at 3 to 4 µm, and dried for 1 h at 65°C. Sections were then immersed in xylol, ethanol, and distilled water. Finally, sections were stained with hematoxylin and eosin and mounted in hydrophobic resin.

RESULTS

Vaccine construction and expression of recombinant M proteins. The six vaccine strains expressing M protein peptides of emm types 1, 2, 4, 9, 12, and 28 were constructed as described in Materials and Methods (Fig. 1). As shown in Fig. 2, a protein of the expected size is overexpressed by each of the 6 vaccine strains in the presence of 20 ng/ml of nisin. In order to verify that the protein was cell associated, the supernatants of the culture media were precipitated with TCA and analyzed by SDS-PAGE in the same way as the cell pellets. No proteins were observed in the supernatants, indicating that the proteins are cell associated (data not shown).

L. lactis expressing the M9 protein (L. lactis M9 strain) promotes M9-specific antibody production. Mice were immunized intranasally with the L. lactis M9 strain or L. lactis WT or were unimmunized (PBS). Specific anti-M9 antibodies were measured by ELISA in sera collected 14 days after the first immunization and boosts. In sera collected 14 days after the first immunization and 14 days after the first boost, the mean antibody titer in mice immunized with the L. lactis M9 strain was higher than that in mice immunized with the L. lactis WT strain or unimmunized mice. However, differences between these groups were not significant. In contrast, the mean antibody titer in sera collected 14 days after the second boost was significantly higher in mice immunized with the L. lactis M9 strain than that in unimmunized mice (P = 0.0397) (Fig. 3). This result indicates that the L. lactis M9 strain vaccine is effective at eliciting specific antibodies after the second boost.

Immunization with the L. lactis M9 strain protects against intranasal challenge with GAS. As shown in Fig. 4A, significant weight loss was observed in unimmunized mice or mice immunized with the L. lactis WT strain after challenge. In contrast, mice immunized with the L. lactis M9 strain showed no significant weight loss after GAS challenge, similarly to uninfected mice (Fig. 4A). Body temperature showed a significant decrease in unimmunized mice and in mice immunized with the L. lactis WT 18 h after the challenge. In contrast, mice immunized with the L. lactis M9 strain did not exhibit a drop in body temperature (Fig. 4B).

One day after challenge, no CFU of GAS were recovered from...
the oropharyngeal washes of mice immunized with the *L. lactis* M9 strain. Figure 4C shows that unimmunized mice have a much higher bacterial count than mice immunized with the *L. lactis* WT strain, whereas mice immunized with the *L. lactis* M9 strain were not colonized with GAS, which suggests that mice vaccinated with the *L. lactis* M9 strain were significantly protected against pharyngeal infection.

Immunization with the *L. lactis* M9 strain prevents histopathological damage in lungs after GAS infection. Lungs of the four groups of mice were examined 6 days after challenge. The histologic examination of lung sections of unimmunized mice showed multiple areas of diffuse and extensive cellular infiltrate with several PMNs that completely disrupted the normal alveolar architecture (Fig. 5A and B). Lung sections from mice immunized with the *L. lactis* WT strain exhibit a similar appearance to unimmunized mice (Fig. 5C and D). At higher magnification (Fig. 5, right panel), it can be observed that the cellular infiltrate is formed by PMNs (indicated with asterisks in Fig. 5B and D). In contrast, lung sections from mice vaccinated with the *L. lactis* M9 strain (Fig. 5E and F) exhibited a normal histopathologic appearance, similar to uninfected mice (Fig. 5G and H). The histopathological score (Fig. 5I) is in accordance with the lung sections shown.

**DISCUSSION**

The *L. lactis* M9 vaccine strain evoked an M9 peptide-specific systemic antibody response when delivered intranasally. The serum antibody titer after 3 immunizations was similar to that obtained with multivalent fusion protein vaccines (20–22). Although we did not determine mucosal levels of IgA in saliva, we were unable to recover GAS from the oropharynx of vaccinated mice, suggesting that immunization impacted GAS colonization or infection at the level of the mucosa. Whether this was mediated by secretory IgA or IgG that reached the mucosa via passive mechanisms is unclear. The ideal GAS vaccine would evoke protective immune responses at both the mucosal and systemic levels. Antibodies that block attachment, colonization, or mucosal infection would serve as the first level of protection (23). Serum opsonic
antibodies that promote bactericidal killing of GAS once local or systemic invasion has occurred would provide the next level of protection against acute illness and potentially serious, invasive disease (24). We have generated 5 additional \textit{L. lactis} vaccine strains to be used as mucosal vaccines against GAS \textit{emm} types 1, 2, 4, 12, and 28. Future studies will evaluate the mucosal immune responses to M peptides delivered via the \textit{L. lactis} vector.

It is interesting that mice vaccinated with the \textit{L. lactis} WT strain exhibit a slight decrease in oropharyngeal colonization with GAS and cellular infiltration in the lungs, which did not correlate with protection against infection because mice immunized with the \textit{L. lactis} WT strain exhibited a pronounced loss of body weight and exhibit a slight decrease in oropharyngeal colonization with GAS and cellular infiltration in the lungs, which did not correlate with protection against infection because mice immunized with the \textit{L. lactis} WT strain exhibited a pronounced loss of body weight and
temperature drop after challenge. These results suggest that specific anti-M9 antibodies were responsible for the protection observed in mice immunized with the L. lactis M9 strain. It is possible that the L. lactis vector expresses surface proteins that evoke antibodies that cross-react with GAS proteins. In fact, sequence homology between specific genes reveals that L. lactis is more closely related to S. pyogenes than to certain other lactococci (25). If future studies define these cross-reactive proteins, the results may suggest that the food-grade vector has potential added immunological value in vaccine design. On the other hand, despite the fact that M protein is rarely detected in the growth media of GAS, a protective effect by the M6 MAD cannot be discarded, and it would be very interesting to determine if there is specific immunogenicity against this portion of the M molecule.

Vaccines based on recombinant bacteria expressing heterologous antigens are potentially a lower cost alternative to those comprised of purified recombinant proteins, especially for low- and medium-income countries. Live-vecorted vaccines require less complex processing and lower production costs than purified proteins. LAB are good candidates because they are “generally regarded as safe” (26). In fact, clinical and microbiological responses to Streptococcus gordonii, a commensal LAB, have already been assessed in healthy volunteers, which demonstrated that its use as a live mucosal vaccine vector is feasible (27). LAB vaccines can be administered intranasally, which may prove to be the optimal immunization route for a GAS vaccine (20, 28). The vaccine candidate presented here can be produced inexpensively and easily delivered without the need for a syringe or highly skilled personnel. The concept of producing one transformed vector for each prevalent emm type of GAS would also address the issue of geographic variation in the epidemiology of these infections. Vaccines formulated for one geographic area could be modified according to epidemiologic data in a different population without major modifications to the manufacturing process. We believe the results presented in the present study provide a proof of principle that could potentially result in a new and different approach to GAS vaccine design that addresses a pressing global need.

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