Mucosal Vaccine Made from Live, Recombinant *Lactococcus lactis* Protects Mice against Pharyngeal Infection with *Streptococcus pyogenes*

Praveen Mannam,† Kevin F. Jones,1,2 and Bruce L. Geller1*

Department of Microbiology, Oregon State University,1 and Siga Technologies, Inc.,2 Corvallis, Oregon

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A novel vaccine (LL-CRR) made from live, nonpathogenic *Lactococcus lactis* that expresses the conserved C-repeat region (CRR) of M protein from *Streptococcus pyogenes* serotype 6 was tested in mice. Nasally vaccinated mice produced CRR-specific salivary immunoglobulin A (IgA) and serum IgG. Subcutaneously vaccinated mice produced CRR-specific serum IgG but not salivary IgA. A combined regimen produced responses similar to the salivary IgA of nasally vaccinated mice and serum IgG of subcutaneously vaccinated mice. Mice vaccinated nasally or with the combined regimen were significantly protected against pharyngeal infection following a nasal challenge with *S. pyogenes* M serotype 14. Mice vaccinated subcutaneously were not protected against pharyngeal infection. Mice in all three LL-CRR vaccination groups were significantly protected against the lethal effects of *S. pyogenes*. Only 1 of 77 challenged mice that were vaccinated with LL-CRR died, whereas 60 of 118 challenged mice that were vaccinated with a control strain or phosphate-buffered saline died. In conclusion, mucosal vaccination with LL-CRR produced CRR-specific salivary IgA and serum IgG, prevented pharyngeal infection with *S. pyogenes*, and promoted survival.

The Centers for Disease Control and Prevention estimates that group A streptococci (*Streptococcus pyogenes*) cause 7 to 20 million cases of pharyngitis in the United States each year, mostly in children under the age of 10. Up to 5% of individuals with untreated or ineffective treated streptococcal pharyngitis can expect to contract acute rheumatic fever (8). More serious sequelae or other types of group A streptococcal infections occur less frequently, such as glomerulonephritis or necrotizing fasciitis, but can be life-threatening (47). Although antibiotics can control streptococcal pharyngitis, painful sore throats would be avoided if an effective vaccine could be developed.

The M6 protein of *S. pyogenes* is a virulence factor that imparts antigenic diversity and plays a central role in the pathogenesis of group A streptococcal infections. It facilitates colonization of mucosal surfaces and enables the organism to escape host immune surveillance (22, 24, 27, 30). Fischetti and colleagues have identified a region of the M protein that is repeated within the protein and conserved among many, if not all, serotypes (C-repeat region [CRR]) (25, 26). Importantly, CRR-specific secretory immunoglobulin A (IgA) but not systemic IgG protects animals against streptococcal pharyngeal infections at the mucosal point of entry, as judged by a reduction in pharyngeal infection following nasal challenge (2, 3, 5, 15, 16).

Commensal and nonpathogenic bacteria are being developed as mucosal vaccine delivery vehicles (34, 35, 36, 40, 49, 52). Risk of infection is low, which is advantageous, particularly for children, the elderly, or immunocompromised individuals.

*Lactococcus lactis* is a nonpathogenic, non-spore-forming gram-positive bacterium that was originally isolated from milk and surfaces of plants and is now used in the dairy industry to make cheese and other fermented foods (33). It is generally recognized as safe by the U.S. Food and Drug Administration. Many heterologous proteins have been expressed in *L. lactis* (11, 12, 18, 23, 31, 42, 53), and immunization with these strains elicits immune responses specific to heterologous antigens (43, 52, 55). However, we are aware of only one report (55) that shows that mucosal immunization with a lactococcal vaccine can reduce infection. We now report that mice immunized mucosally with a strain of *L. lactis* that expresses an M protein antigen were protected against pharyngeal infection following a challenge with *S. pyogenes*.

**MATERIALS AND METHODS**

**Bacterial strains and media.** *L. lactis* LM2301(pP16pipM6c), which expresses CRR (LL-CRR), and *L. lactis* LM2301(pP16pipM6cT14) (M serotype 14; Rockefeller University Culture Collection) was grown in Todd-Hewitt broth with 1% yeast extract and 200 μg of streptomycin/ml and plated on Todd-Hewitt plates with 1% yeast extract, 5% defibrinated sheep blood (Cleveland Scientific, Bath, Ohio), and 200 μg of streptomycin/ml.

**Immunization protocol.** Preimmune saliva and serum samples were collected from 4-week-old CD1 Swiss-Webster female mice (Charles River Laboratories, Wilmington, Mass.) as described below. Mice were vaccinated nasally under 5% isoflurane anesthesia by instilling into both nostrils on 3 consecutive days 20 μl of PBS or a cell suspension containing a total of either 1 × 10^9 or 2 × 10^9 CFU/ml. Mice were vaccinated subcutaneously by injecting in the interscapular region 100


µl of either PBS or a cell suspension containing 5 × 10^8 CFU. Mice vaccinated with a combined regimen received both the nasal and subcutaneous doses on the first day, followed by only the nasal dose on the two consecutive days. This schedule was repeated beginning 14 and 28 days later. Fourteen days after the last vaccination, saliva and blood samples were collected.

**Sample collection.** Blood samples were collected from a tail vein, incubated for 1 h at 37°C, and centrifuged at 1,500 × g for 10 min. The serum was separated and stored at −20°C. Saliva was collected using pilocarpine and bonded polyester wicks (Filtra, Richmond, Va.) as described previously (39), diluted into 300 µl of saliva processing solution (0.5% bovine serum albumin, 0.02% NaN₃, and 1× complete protease inhibitor [Boehringer, Mannheim, Germany] in PBS), mixed, centrifuged (10,000 × g, 5 min), and stored at −20°C. Dilution of saliva by this method was about 0.25, as measured by the decrease in A₅₅₀ of a sample of bovine serum albumin processed in parallel with the saliva.

**ELISA.** CRR-specific IgA in saliva and IgG in serum were quantified by enzyme-linked immunosorbent assay (ELISA) as described previously (1, 34). Black, 96-well microplates (Packard, Meriden, Conn.) were coated with purified, recombinant M₆ protein. Standard curves were established on every plate with use of a twofold dilution series of mouse IgA or mouse IgG (Sigma Chemical Co., St. Louis, Mo.), Goat anti-mouse IgA (Sigma) and IgG (Bethyl, Montgomery, Tex.) conjugated to horseradish peroxidase and chemiluminescent substrate (SuperSignal ELISA; Pierce, Rockford, Ill.) were used to develop the signal, which was read in a Wallac 1450 MicroBeta TriLux counter (Wallac, Turku, Finland). Concentrations of M₆-specific IgA and IgG were extrapolated from the standard curves.

**Passage and titration of challenge strain.** Streptococmycin-resistant S. pyogenes T14 was passed nine times in groups of five Swiss CD1 mice and titrated for pharyngeal infection in 50 to 75% of the mice as described previously (2).

**Challenge of vaccinated mice.** Vaccinated mice under 5% isoflurane anesthesia were challenged with 20 µl (6 × 10⁸ CFU) of S. pyogenes T14 instilled into both nostrils. Throats were swabbed on days 4, 5, 7, 9, and 11 postchallenge, and swabs were cultured as described previously (2). Cultures displaying one or more beta-hemolytic colonies were scored as positive. All procedures involving animals were performed in compliance with federal and state laws and guidelines and approved by the Oregon State University Institutional Animal Care and Use Committee (approval no. 2777).

**Statistical analysis.** Data were analyzed using GraphPad Instat software, version 3.05 (San Diego, Calif.). The Mann-Whitney test was used to compare the mean salivary IgA and serum IgG responses in the different experimental groups. Group means were calculated by including all individual values. Variance is expressed as standard error of the mean. Fisher’s exact test was used to analyze proportions of mice infected and dead among groups of mice in the challenge experiment and infected or not among nasally vaccinated mice with or without an IgG response. P values of less than 0.05 were considered significant. P values of less than 0.01 were considered highly significant.

**RESULTS**

**Vaccination.** Groups of mice (Table 1) were vaccinated nasally (group 1) or subcutaneously (group 4) or by a combined regimen (nasal plus subcutaneous, group 6) with L. lactis that expresses the CRR of the M serotype 6 protein on its cell surface (LL-CRR). Control mice (groups 2, 3, 5, and 7) were vaccinated in an identical way with either PBS or an isogenic strain of L. lactis that does not express CRR (LL). Vaccination was repeated twice, 14 and 28 days after initial vaccination. Two weeks after the final vaccination, blood and saliva were collected and analyzed by ELISA for CRR-specific responses.

**CRR-specific salivary IgA.** All nasally vaccinated mice (group 1, LL-CRR) produced a CRR-specific salivary IgA response, with a mean of 0.88 ng/ml (Fig. 1). Only one and four mice in control groups 2 (LL) and 3 (PBS), respectively, had a barely detectable response. Differences between groups 1 and 2 or 3 are highly significant (P < 0.001).

Subcutaneously vaccinated mice (groups 4 [LL-CRR] and 5 [LL]) did not produce significant (P > 0.1) CRR-specific salivary IgA (Fig. 1). Seven and two mice in groups 4 and 5, respectively, had barely detectable levels of CRR-specific salivary IgA.

All mice in combined regimen group 6 (LL-CRR) produced a CRR-specific salivary IgA, with a group mean of 1.21 ng/ml. Control group 7 (LL) did not produce a significant response. The difference in response between groups 6 and 7 is highly significant (P < 0.0001).

**CRR-specific serum IgG.** CRR-specific serum IgG was detected in seven mice from the nasally vaccinated group 1 (LL-CRR), and the treatment group mean response was 19.98 ng/ml (Fig. 2). Five mice in control group 2 (LL) responded, and the treatment group mean response was 6.96 ng/ml. The difference in response between these groups was not statistically significant (P = 0.24). Only one mouse in control group 3 (PBS) showed a response of 6.22 ng/ml.

Sixteen mice vaccinated subcutaneously (group 4, LL-CRR) produced a strong CRR-specific serum IgG response, with a mean of 57.41 ng/ml (Fig. 2). Six mice in control group 5 (LL) responded with a mean of 7.19 ng/ml. The difference in responses between groups 4 and 5 is highly significant (P < 0.001).

All mice in combined regimen group 6 (LL-CRR) showed a
highly significant \( P \leq 0.001 \) than that of group 1 but not of group 4 \( (P = 0.49) \).

**Effect of increased dosage.** Eighteen mice (Table 1, group 8) were nasally vaccinated with a fourfold-higher dosage of LL-CRR to determine if this would increase the immune responses. All mice responded with a CRR-specific salivary IgA response, and the group mean was 0.74 ng/ml (Fig. 1). This was not significantly different from the response of the lower-dosage group 1. Three mice in each of two control groups (9 and 10) given either an equivalent higher dosage of LL or PBS produced low-level CRR-specific salivary IgA. Control group 9 and 10 means were 0.003 and 0.064 ng/ml, respectively, which were significantly \( P < 0.0001 \) lower than the response from group 8.

Six mice in the high-dose nasal group 8 had detectable CRR-specific serum IgG responses, and the group mean was 18.84 ng/ml. There is no significant difference between the CRR-specific serum IgG responses in groups 1 and 8. Three mice in control group 9 showed a response with a mean value of 4.33 ng/ml, which was not significantly different from group 8. Only two mice in group 10 showed a response (mean of 5.66 ng/ml), which was not significantly lower than that of group 8.

**Challenge with S. pyogenes.** All mice were challenged nasally with an infectious dose of S. pyogenes M serotype 14. Throat swabs were taken 4, 5, 7, 9, and 11 days postchallenge. Mice were scored positive for pharyngeal infection if on any day one or more beta-hemolytic colonies were detected.

Mice vaccinated nasally or with the combined regimen were significantly protected against pharyngeal infection (Fig. 3). Five mice vaccinated nasally with LL-CRR (group 1) were infected, compared to 14 and 13 mice in control groups 2 (LL) and 3 (PBS), respectively. This difference is statistically significant \( P = 0.010 \) and 0.025, respectively). Four mice in combined regimen group 6 (LL-CRR) were infected, whereas 13 mice in its control group 7 (LL) were infected, which is a significant \( P = 0.008 \) difference.

Subcutaneously vaccinated mice were not significantly protected from pharyngeal infection. Nine mice in group 4 (LL-CRR) were infected, compared to 15 in its control group 5 (LL), which is not statistically different \( P = 0.105 \).

Mice vaccinated nasally with the higher dosage of LL-CRR were also significantly protected from pharyngeal infection. Five mice in group 8 (LL-CRR) were infected, compared to 14 and 13 in its control groups 9 (LL) and 10 (PBS), respectively. These differences are statistically significant \( P = 0.0176 \) and 0.0437, respectively.

**Analysis of mortality.** Comparison of mortality of the challenged mice shows only one death (in group 8) among the 77 total mice vaccinated with LL-CRR (Table 2). In contrast, mice treated with LL or PBS showed 49 and 55% mortality, respectively. Fisher’s exact test revealed that differences in mortality rates between the mice vaccinated with L. lactis CRR and control groups were highly significant \( P < 0.001 \). There

### Table 2. Mortality

<table>
<thead>
<tr>
<th>Group no.</th>
<th>Type(^a)</th>
<th>No. of deaths/total no.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Nasal+SubQ LL-CRR</td>
<td>0/20</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Nasal+SubQ LL</td>
<td>11/20</td>
<td>55</td>
</tr>
<tr>
<td>3</td>
<td>Nasal+SubQ PBS</td>
<td>10/20</td>
<td>50</td>
</tr>
<tr>
<td>4</td>
<td>SubQ+SubQ LL-CRR</td>
<td>0/20</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>SubQ+SubQ LL</td>
<td>12/20</td>
<td>60</td>
</tr>
<tr>
<td>6</td>
<td>Nasal+SubQ LL</td>
<td>0/19</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>Nasal+SubQ PBS</td>
<td>6/18</td>
<td>33</td>
</tr>
<tr>
<td>8</td>
<td>Nasal Hi LL-CRR</td>
<td>1/18</td>
<td>6</td>
</tr>
<tr>
<td>9</td>
<td>Nasal Hi LL</td>
<td>9/20</td>
<td>45</td>
</tr>
<tr>
<td>10</td>
<td>Nasal Hi PBS</td>
<td>12/20</td>
<td>60</td>
</tr>
</tbody>
</table>

\(^a\) SubQ, subcutaneous; Nasal Hi, higher-dose groups.
were no statistically significant differences in mortality among the different control groups.

**Correlation between immune responses and protection from pharyngeal infection.** The relationship between CRR-specific immune responses and protection against pharyngeal infection was analyzed further. All 195 mice in the study, including experimental and control groups, were separated into pharyngeally infected and noninfected groups (Table 3). Differences between these two groups in mean CRR-specific salivary IgA and serum IgG were both significant ($P < 0.0001$ and $P = 0.0130$, respectively).

The role of CRR-specific serum IgG in prevention of pharyngeal infection was analyzed further. Mice vaccinated subcutaneously (group 4, LL-CRR), none of which showed a CRR-specific salivary IgA response, were grouped for analysis according to the results of pharyngeal infection. The mean CRR-specific serum IgG values in the pharyngeally infected and noninfected groups were 46.06 and 75.37 ng/ml, respectively, which is not significantly different ($P = 0.44$). This suggests that the correlation between protection from pharyngeal infection and CRR-specific serum IgG shown by the analysis of all 195 mice (Table 3) may not indicate a cause-and-effect relationship.

The roles of CRR-specific serum IgG and salivary IgA in pharyngeal infection were analyzed further. Mice from nasal vaccination groups 1 (low-dose LL-CRR) and 8 (high-dose LL-CRR), all of which responded with CRR-specific salivary IgA, were divided into two groups for analysis: those with and those without CRR-specific serum IgG responses. Of those with a CRR-specific serum IgG response ($n = 13$), three had pharyngeal infections (23.1%). Of those without a CRR-specific serum IgG response ($n = 25$), seven had pharyngeal infections (28.0%). The proportion of each group that was infected is not significantly different ($P > 0.1$). This suggests that protection from pharyngeal infection was independent of CRR-specific serum IgG response and that CRR-specific salivary IgA response may be sufficient for protection.

**Correlation between immune responses and survival.** All 195 mice were also grouped using survival as the sole criterion and then analyzed for mean CRR-specific immune responses (Table 3). Survivors had significantly higher CRR-specific salivary IgA or serum IgG levels than those that died ($P < 0.0001$ and $P = 0.0018$, respectively).

We eliminated from analysis all mice that did not have a pharyngeal infection. The 106 mice that had a pharyngeal infection were grouped according to survival and then analyzed for group mean immune responses (Table 3). Survivors had significantly higher CRR-specific salivary IgA or serum IgG levels than those that died ($P = 0.0008$ and $P = 0.0205$, respectively).

**DISCUSSION**

Development of a vaccine against streptococcal pharyngitis is challenging for a number of reasons. Over 120 serotypes have been identified (13, 30), which makes it difficult to find conserved epitopes that could be used to elicit cross-serotype protection. The CRR of M protein is one such epitope (2, 5, 15, 25). However, CRR-specific secretory IgA and not serum IgG is required for prevention of pharyngeal infection with *S. pyogenes* (2, 3). Evidence suggests that production of antigen-specific secretory IgA is best achieved by delivery of antigen on particulates (7, 17, 38, 52) to avoid oral tolerance associated with soluble antigens (9, 37, 48, 54) and requires presentation of the antigen directly to the mucosal surface (29). The only approved mucosal vaccines are pathogens (49), some of which may pose a risk of infection, particularly to children or immuno-compromised individuals (36, 39, 46). Therefore, recent efforts to develop mucosal vaccine delivery systems have included ones that are not pathogenic, such as liposomes or microparticles (10, 50), commensal bacteria (19, 20, 32, 34, 39, 45, 46), or noninvasive, noncommensal bacteria (11, 18, 31, 42).

This report shows that nasal vaccination with a live strain of *L. lactis* prevented pharyngeal infection with *S. pyogenes*. Moreover, this protection was cross-serotype. An analysis of all 195 mice grouped according to the results of pharyngeal infection showed a correlation between protection from pharyngeal infection and either CRR-specific salivary IgA or serum IgG. This suggested that one or perhaps both responses were sufficient for protection from pharyngeal infection. Further analyses performed by grouping mice with only an IgG or IgA response revealed a correlation between protection from infection and CRR-specific salivary IgA but not CRR-specific serum IgG. These results are consistent with previous results that show that M6-specific IgA prevents adhesion to epithelial cells in vitro whereas M6-specific IgG does not (16), passive administration of M6-specific IgA reduces pharyngeal infection in mice (3), and immunization with CRR prevents pha-

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**TABLE 3. Correlation of antibody response and protection from pharyngeal infection (colonized)**

<table>
<thead>
<tr>
<th>Groupinga</th>
<th>n</th>
<th>Saliva IgA concn (ng/ml)</th>
<th>$P$</th>
<th>Serum IgG concn (ng/ml)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colonized</td>
<td>105</td>
<td>0.09115</td>
<td>&lt;0.0001</td>
<td>11.352</td>
<td>0.0130</td>
</tr>
<tr>
<td>Not colonized</td>
<td>90</td>
<td>0.5695</td>
<td></td>
<td>28.436</td>
<td></td>
</tr>
<tr>
<td>Mice that died during challenge</td>
<td>61</td>
<td>0.04129</td>
<td>&lt;0.0001</td>
<td>5.987</td>
<td>0.0018</td>
</tr>
<tr>
<td>Mice that survived challenge</td>
<td>134</td>
<td>0.4197</td>
<td></td>
<td>24.480</td>
<td></td>
</tr>
<tr>
<td>Colonized, survived</td>
<td>45</td>
<td>0.1591</td>
<td>0.0008</td>
<td>17.248</td>
<td>0.0205</td>
</tr>
<tr>
<td>Colonized, died</td>
<td>60</td>
<td>0.04101</td>
<td></td>
<td>4.974</td>
<td></td>
</tr>
</tbody>
</table>

$a$ All the mice in the study were grouped using pharyngeal infection (colonized), mortality, or mortality after pharyngeal infection as a criterion. The mean salivary IgA and serum IgG concentrations were calculated and compared. $P$ values are indicated beside each group.
rhyeal infection (2, 5). Our results differ from those in a previous report that showed protection against pharyngeal infection without measurable CRR-specific salivary IgA in mice vaccinated with a recombinant vaccinia virus that expresses CRR (15). Our results suggest that CRR-specific salivary IgA was both necessary and sufficient to prevent pharyngeal infection with *S. pyogenes*. A potential role for cell-mediated immunity is the subject of future investigations. A contribution from CRR-specific serum IgG to prevention of pharyngeal infection cannot be eliminated, as a trend in that direction is apparent (Fig. 3). A larger treatment group may be necessary to investigate this further.

The mechanism by which M-specific secretory IgA prevents colonization in mucosally vaccinated animals is unknown. Although M- or CRR-specific secretory IgA is not opsonic (2, 4), it blocks adhesion to epithelial cells and protects against streptococcal infection (3, 16). In comparison, passive, intranasal administration of M-specific IgG does not significantly reduce infection (3) nor reduce adhesion to epithelial cells in vitro (16). This may suggest that differences in the immunoglobulin structures play a role in the mechanism by which M-specific secretory IgA prevents colonization.

A combination of subcutaneous vaccination followed by nasal vaccination was examined to determine if the former would “prime” the latter and lead to an elevated level of CRR-specific salivary IgA. The results show that CRR-specific salivary IgA levels were significantly higher (*P* = 0.024) in the group vaccinated with the combination regimen than in the group vaccinated only nasally. This suggests that subcutaneous vaccination with *L. lactis*, although by itself not producing a mucosal response, increases the mucosal immune response to mucosally administered antigens, at least when delivered in live *L. lactis*.

As expected, subcutaneous delivery of the vaccine elicited significantly higher CRR-specific serum IgG levels than did nasal delivery. However, the increased levels did not translate to a higher level of survival. Apparently nasal delivery elicits adequate levels of antigen-specific serum IgG, or CRR-specific salivary IgA contributes to survival (see discussion below).

The effect of dosage on efficacy showed that increasing the nasal dose fourfold did not significantly change the CRR-specific salivary IgA or serum IgG level compared to that with the lower dosage. Although the level of protection against pharyngeal infection trended higher, it was not statistically different from that of the lower-dosage group. This suggests that adequate protection might be achieved with dosages even lower than those used here. Future studies are planned to optimize the number of doses required for adequate protection and to determine the duration of protection.

Another finding was that mucosal vaccination with LL-CRR promoted survival and prevented death. It is known that M-specific serum IgG opsonizes *S. pyogenes* or serum IgG level compared to that with the lower dosage. Although the level of protection against pharyngeal infection trended higher, it was not statistically different from that of the lower-dosage group. This suggests that adequate protection might be achieved with dosages even lower than those used here. Future studies are planned to optimize the number of doses required for adequate protection and to determine the duration of protection.

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