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Praveen Mannam for the degree of Master of Science in Microbiology presented on June 4, 2003.

Title: Immune Response and Protection against *Streptococcus pyogenes* after Vaccination with *Lactococcus lactis* that expresses conserved region of M6 protein.

Abstract approved:

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Bruce L. Geller

Most pathogens gain access to their host through mucosal surfaces. It is therefore desirable to develop mucosal vaccines that elicit an immune response to prevent this crucial first step in infection. Current mucosal vaccines are live attenuated strains of pathogens. More recent efforts have focused on the use of recombinant non-pathogenic gram-positive bacteria as live vaccine delivery vectors. Here I have tested the potential of *Lactococcus lactis* to be used as a vaccine vector. A recombinant strain of *L.lactis* has been constructed which expresses and displays on its surface the C repeat region (CRR) of the M6

protein of *Streptococcus pyogenes*. I show that nasal vaccination of mice with this strain elicited strong salivary IgA and serum IgG response. These responses protected mice against a nasal challenge with *S.pyogenes*. Subcutaneous vaccination with the same strain of *L.lactis* produced a strong serum IgG response, but no salivary IgA response. Subcutaneous vaccination did not protect the mice against nasal infections when the mice were challenged with *S.pyogenes*. The immune response and protection afforded by concomitant vaccination by both nasal and subcutaneous routes were better that that seen in nasal vaccination alone. This study shows that an effective vaccine against *S.pyogenes* is possible using *L.lactis* as a vaccine vector. It also opens up the potential of *L.lactis* to be used in the development of vaccines to other mucosal infections.

Immune Response and Protection against *Streptococcus pyogenes* after Vaccination with *Lactococcus lactis* that expresses conserved region of M6 protein.

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INTRODUCTION

Many bacterial infections begin with colonization of mucosal surfaces. The mucosal site represents a major portal of entry for pathogens. The mucosal surfaces are protected both by non-specific mechanisms, like production of mucous, lysozyme, lactoferrin, lactoperoxidase etc and specific immune mechanisms mediated by the lymphoid tissue. The principal inductive sites that operate on the mucosal surface are Gut associated lymphoid tissue (GALT) and Bronchial associated lymphoid tissue (BALT). Recent studies in mice have revealed the presence of Nasal associated lymphoid tissue (NALT). The common features of all these inductive sites include the presence of M cells on the epithelial surface and also plasma cells, dendritic cells, macrophages, glandular cells, CD4+ and CD8+ T cells. The main antibody type involved in mucosal immunity is S-IgA present in saliva, tears, gastrointestinal fluid, urine and other secretions (59). S-IgA is the most abundant immunoglobulin in the body with 75% of the antibody-producing cells devoted to its production (42). S-IgA acts by prevention of adsorption of pathogens and toxins at the epithelial surface. S-IgA also neutralizes intracellular microbial pathogens, such as viruses, directly within epithelial cells and binds to antigens in the mucosal lamina propria and excretes them through the adjacent epithelium into the lumen (42). Development of

antigen specific S-IgA antibodies at the mucosal surfaces often confers immunity against infection.

There is evidence that presentation of an antigen at a specific inductive mucosal site results in higher levels of IgA at that site than at distant mucosal locations. B cells undergo isotype switching at inductive sites of the mucosa. The activated S-IgA producing B cells migrate to the lamina propria of the effector sites in the mucosal tissue with a preponderance of homing to the original site of antigenic exposure (43,45). This compartmentalization of the common mucosal immune system (CMIS) means that the most effective way of stimulating an immune response at a particular site is through localized presentation of antigens at that site (37,47).

Oral administration of an antigen can result in stimulation of local and systemic immunity. It can also result in tolerance to that antigen (49,26). This represents the dichotomy of the mucosal immune system, which can tolerate beneficial antigens present in food and harmless antigens presented by the commensal bacteria while mounting a specific immune response against the antigens present on pathogenic bacteria. Smith *et al* have shown that presentation of soluble antigen (ovalbumin) alone produces tolerance while ovalbumin administered along with a strong adjuvant like cholera toxin primed the immune cells (56). Administration of soluble protein produces tolerance or at the most a weak immune response. Antigens are much more immunogenic when presented in inert particles or displayed on the surface of bacteria (15,13).

The available mucosal vaccines, such as the bacterial vaccines against typhoid or cholera and the viral ones against polio or rotavirus, use live attenuated pathogens (18). However there are safety and environmental issues involved in the use of engineered pathogens. There is a concern about reversion of the attenuated strains to virulence. People with either congenital or acquired immunodeficiency diseases should not be administered Oral polio vaccine (OPV) because of the risk of paralytic disease. In very rare cases OPV has been associated with incidence of paralytic polio in healthy recipients and their contacts (58). In 1999 CDC recommended the withdrawal of rhesus rotavirus vaccine-tetravalent (RRV-TV) (RotaShield, Wyeth Laboratories, Inc., Marietta, Pennsylvania) because of increased risk of intussusception in some infants within 30 days of immunization (9,10).

Non-pathogenic Gram-positive bacteria such as *S. gordonii* and *L.lactis* are being investigated as alternative vaccine vectors. *L.lactis* is used in the diary industry to make cheese and sour cream. It can be safely consumed by humans and has been designated GRAS (generally regarded as safe) by the Food and drug administration (27). *L.lactis* can be grown easily in the lab and a number of bacterial, viral and parasitic antigens have been efficiently expressed by *L.lactis* (61,11,22,62,32). It can survive passage through the gastrointestinal tract (38, 19) and, unlike *S.gordonii,* is non-colonizing (38,31). *L.lactis* has an immunoadjuvant effect on the gut immune system (11,50). This effect can be enhanced by the co expression of cytokines such as IL-2 and IL-6 along with the

foreign antigen (57). Thus *L.lactis* is an ideal candidate for an antigen delivery vehicle.

Oral immunization with recombinant *L.lactis* expressing the surface protein antigen (Pac) from *Streptococcus mutans* serotype c produced a significant salivary IgA and serum IgG response in mice (32). *L.lactis* have been used successfully stimulate a mucosal immune response against the tetanus toxin fragment C and protect against subsequent challenge (61). Similar studies have shown the effectiveness of *L.lactis* in producing a specific immune response against Env protein of HIV (62) and urease B subunit of *H.pylori* (41). Enouf *et al* have shown that L.Lactis is able to express and elicit an immune response to rotavirus non-structural protein (NSP4) (22). In this study we examined the ability of *L.lactis* expressing a part of the M6 protein of *Streptococcus pyogenes* to generate a specific and protective immune response against the pathogen.

S.pyogenes (Group A streptococcus) is a common pathogen that colonizes the throat and skin of humans. It usually causes mild illness such as impetigo or pharyngitis. It can also cause serious infections such as Toxic shock syndrome, nectotizing fascitis (14). Its post infectious sequelae like rheumatic heart disease and glomerulonephritis are a serious health problem in third world countries (2). Even in United States there has been a resurgence of Group A streptococcal cases since the mid 80's (35). Although antibiotics can control streptococcal infections, there is a need to develop an effective vaccine. Long-term prophylactic antibiotic treatment is indicated after rheumatic fever and

rheumatic heart disease to prevent further damage to heart valves (30,54). Vaccines are a safe, convenient and cheap alternative.

The major virulence factor of *S.pyogenes* is M protein, an alpha helical coiled-coil bacterial surface protein (40). It is involved in the initial attachment of bacteria to the pharyngeal and dermal epithelial cells (20,21). M protein binds to complement control factors and other host proteins to prevent activation of the alternate complement pathway and thus evade phagocytosis and killing by polymorphonuclear leucocytes (7,52). More than 80 different antigenic types of M protein have been identified according to serological typing developed by Lancefield (40). Development of type specific antibodies to the M protein confers resistance to streptococcal infection (40). Attempts to create an effective vaccine against *S.pyogenes* have been based on the M protein. These attempts have been hampered by the fact that there are a large number of M types. The C-terminal part of the M protein is conserved among strains of different serotypes while the N-terminal part shows variability (24). This conserved region consists of a series of repeats and is called the C repeat region (CRR). An immune response directed against the epitopes in this region can protect against colonization and death following streptococcal challenge in mice (4,6). The CRR of M protein is a good candidate for a vaccine against *S.pyogenes*.

Acute Rheumatic fever (ARF) is postinfectious sequelae of Group A streptococcal (GAS) pharyngitis brought about by an autoimmune reaction to epitopes on the organism that cross react with tissues in heart, joints, brain and skin (36,63). The M protein has been implicated as a source of this cross-

reactive immunity as it structurally and immunologically resembles host tissue antigens such as sarcolemmal protein and myosin in the heart tissue (55,16,39). Although there are a variety of M serotypes only a few are associated with ARF. M5 is the most commonly associated serotypes followed by M3, 6, 14, 18, 19, 24, and a few others. Types such as M2, 4, and 12 are not associated with rheumatic fever (14). A classification method proposed by Bessen *et al* groups the M protein based on the expression of a certain epitope (Type I) which is localized to a 15 amino acid fragment in the C repeat region. The sera of ARF patients contain a high level of antibodies to the Type I epitope (3). It was shown by Mori *et al* that sera from ARF patients had a higher reactivity to C region of the M protein when compared to sera from patients with uncomplicated pharyngitis (48). However in this study the pharyngitis patients were exposed to a much shorter time period than the ARF patients. In a more recent study Jones K F *et al* have shown that the anti M antibodies are not significantly higher in sera of ARF patients when compared to sera of patients with scarlet fever or pharyngitis when both sets of patients had an equivalent exposure to S.pyogenes (34). Also the antibodies against the conserved region of M6 show reactivity to denatured rather than native myosin (60). Thus there is no convincing evidence that the CRR plays a role in the pathogenesis of RHD inspite of the presence of cross-reactive epitopes.

The CRR of the M protein has been genetically fused to Pip protein of *L.lactis* and successfully expressed from a high copy number plasmid (29). Pip is a surface expressed protein. Its physiological role is unknown but it serves as a

receptor for bacteriophage (28). The CRR from nucleotide 823 to 1131 was cloned into the *pip* gene. The recombinant fusion protein was displayed on the surface of *L.lactis* and the M part of the fusion protein was detected using anti-M6 10F5 monoclonal antibody (33). In this study I investigated the immunogenicity in mice of this recombinant *L.lactis* I compared the effectiveness of nasal delivery to parenteral subcutaneous delivery and a combination of both. Finally, I present the results of an infectious challenge of the vaccinated mice with *S.pyogenes*.

MATERIALS AND METHODS

Bacterial strains and media. Two strains of *L.lactis* LM2301 were used in this study. They were *L.lactis* LM2301 (pBG568 pipemmp16) and *L.lactis* LM2301 (pBG568 pipwtp16). These strains express the pip-emm fusion protein and pip protein respectively from a multicopy plasmid with a strong promoter. These strains were constructed previously in the lab as described in Geller *et al* (29). The expression of CRR of the M6 protein and Pip protein was analyzed by immunoblots of intact cells. Recombinant *L.lactis* was grown in M17 medium supplemented with 0.5% glucose (M17G) and 5 μG/ml of erythromycin. An overnight culture was used to inoculate fresh medium at a dilution of 1:25 and the cells were grown to exponential phase (OD600= .5). The cells were then centrifuged, washed twice with sterile PBS and resuspended in sterile PBS to give a final concentrations of 5×10^{10} CFU/ml and 2×10^{11}CFU/ml. *S.pyogenes* T14 (Rockefeller University Culture Collection) was grown in Todd Hewitt broth with 1% yeast extract and plated on Todd Hewitt plates with 1% yeast extract and 5% defibrinated sheep blood (Cleveland Scientific,OH).

Immunoblot analysis. 1.5 milliliter of each culture at an optical density at 600nm (OD_{600}) of .5 was centrifuged at 1300 rpm for 2 min in 1.5 ml microfuge tubes. The cell pellet was washed twice with TBS (20mM TRIS-Hcl-500mM Nacl, pH 7.5). The cells were resuspended in a volume of TBS with adjustment for the differences in OD at the time of harvest. .5 ml of the cell suspension was vacuum

filtered through nitrocellulose membrane (Schleicher & Schuell, N.H) using a slot blot filtration manifold (Hoefer Pharmacia, San Francisco, C.A). The membrane was removed from the apparatus and blocked for 2 hours with TBS containing 3% gelatin and washed 3 times with TTBS (TBS, .01% Teen 20) for 5 min. The membrane was then probed overnight with anti-M6 10F5 monoclonal antibody (55) in TTBS containing 1% gelatin, washed 3 times with TTBS for 5 min and incubated with anti-mouse IgG conjugated to alkaline phosphatase (Bio-rad Laboratories, Hercules, C.A). The signal was developed with Immun-star™ AP substrate pack (Bio-Rad).

Immunization Protocol. Four week-old CD1 female mice were purchased from Charles River laboratories M.A. There were about 20 mice in each experimental group. Pre-immune saliva and serum samples were collected as described below. Nasal vaccinations were done by instilling 20 μL of cell suspension in PBS intranasally into both nostrils under 5% isoflurane anesthesia. Subcutaneous vaccinations were carried out by injection 100 μL of the cell suspension or PBS under the skin in the interscapular region. The mice were vaccinated in three rounds fourteen days apart. Each round consisted of one vaccination on three consecutive days. Fourteen days after the last vaccination saliva and blood samples were collected.

Sample Collection. Blood samples were collected from a vein in the tail. The blood was incubated for 1 hr at 37 C to ensure clot formation and centrifuged at 1500 rpm for 10 min. The serum was separated and stored at –20 C. To collect saliva each mouse was injected with ~100 μG of pilocarpine I.P. The saliva was collected using a 2.5- by 25- mm polyester wick (Whatman, Clifton, N.J) and placed in a 1.5 ml microfuge tube with 300 μL of saliva processing solution (0.5% BSA, 0.02% NaN3 and 1x complete protease inhibitor (Boehringer Mannheim, Germany). The tubes were then vortexed and spun to collect the saliva and stored at –20 C. 1 mg /ml solution of BSA was processed in a similar manner. By measuring the A_{280} of the solution before and after processing we were able to estimate that saliva is diluted 4 times by collection in this method.

ELISA. 96-well black microplates (Packard, Meriden, Conn) were coated overnight at room temperature with 100 μL of M6 protein (5 μG/ml of carbonate buffer). For the standards the wells were coated with 1 μG of anti mouse IgG and IgA per ml of carbonate buffer in a similar way. They were washed the next day with PBS containing .02% sodium azide and 0.05% Brij. They were blocked with 200 μL of casein buffer (5% Casein, 0.05% Brij. pH 7.4) for 2 hours at 37 C. The saliva samples were diluted 1:2 and the serum samples were diluted 1:5 in blocking buffer and incubated (50 μL) in the wells for 3 hours at 37 C. Two fold dilutions of IgA and IgG standards were also incubated in the appropriate wells. After washing the secondary antibodies α MIgG-HRP (Bethyl A90-131P-6) and α MIgA-HRP (Sigma A-4789) were incubated at dilutions of 1:10,000 and 1:2000

respectively for 2 hrs at 37 C. After the final wash, the chemiluminescent substrate (SuperSignal ELISA, Pierce) was added, and the plates read in a Wallac 1450 MicroBeta TriLux counter (Wallac, Turku, Finland). The readings were in LCPS (Luminescence corrected count rate (counts per second) divided by 100). The readings were corrected for background by subtraction of the average reading obtained from blank wells. A standard curve was generated with each plate by graphing the LCPS readings versus the IgG and IgA concentrations. The concentrations of M6-specific IgA and IgG were determined by extrapolating luminescence readings of samples in the standard curve.

Passage and titration of Challenge Strain. The challenge strain of *S.pyogenes* T14 was passaged 9 times in groups of 5 Swiss CD1 mice to enhance the virulence. The mice were injected IP with 100 μL of a 10-fold dilution series of culture gown to an OD_{650} of .6. The spleen of the dead mice that received the highest dilution was harvested and the bacteria were recovered for the next round of passage. After the last passage the recovered bacteria were grown to stationary phase in Todd-Hewitt medium with 5% sheep blood and stored at –70 C.

The nasal dose of *S.pyogenes* required for pharyngeal colonization was determined as follows: Frozen stock of T14 was diluted 1:25 in pre-warmed medium. At OD_{650} of .5, the culture was centrifuged at 13000 RPM for a minute

and the cell pellet was resuspended in PBS at $1/6^{th}$ the original volume. A ten fold dilution series was made starting at 10^{-1}. Each dilution was administered to groups of five mice. Pharyngeal colonization was monitored for eleven days by throat swabs. The dose of *S.pyogenes* that caused approximately 50% colonization was determined to be 6×10^6 CFU. This dose was used in the challenge of immunized mice.

Challenge of Vaccinated mice. Vaccinated mice were challenged with 20 μL (6×10^6 CFU) of *S.pyogenes* instilled into both nostrils under 5% isoflurane anesthesia. The throats of the mice were swabbed and plated onto blood agar plates every day for next four days and then every other day for a total of 11 days. Mice that showed colonization on or after day 4 were scored as positive.

Statistical Analysis. All the data was analyzed using GraphPad InStat software, Version 3.05. The Mann-Whitney test was used to compare the mean salivary IgA and serum IgG response in the different experimental groups. The Fisher's exact test was used to analyze the proportions of mice infected and dead among the groups of mice in the challenge experiment. P-values of less than .05 were considered significant.

RESULTS

Expression of M6-pip fusion protein. The production of the C repeat region of the M6 protein (CRR) by recombinant *L.lactis*, was measured by immunoblots of whole cells from exponential phase cultures. The experimental strain *L.lactis* LM2301 (Pip-CRR) expressed on its surface 6.6 $\mu g/OD_{600}/ml$ (SD = 0.4)of the fusion protein. No specific signal was detected from the control strain *L.lactis* LM2301 (Figure.1). These values are in agreement with previously published results (29).

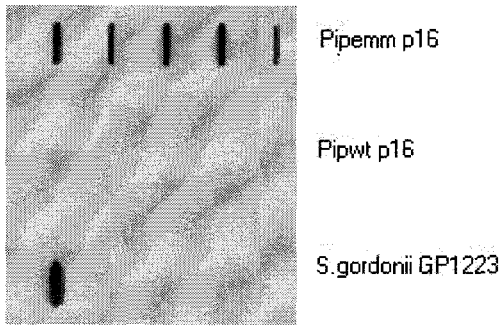

Figure 1. **Immunoblotting.** Recombinant *L.lactis* cells were washed and loaded on a nitrocellulose membrane using a slot blot apparatus. The blot was probed with monoclonal antibody against CRR. The five slots in first row were loaded with *L.lactis* LM2301 (Pip-CRR). The five slots in the second row were loaded with *L.lactis* LM2301. The first slot on row three is loaded with *S.gordonii* (GP 1223) as a positive control for M protein production (12).

Table.1. **Treatment Groups.** The different treatment groups, strains of *L.lactis,* PBS used to immunize the various groups and the routes of immunization. The Average CFU/ml of cells administered in each dose and the number of mice in each group is also indicated.

Group no	Vaccine strain	Route of Vaccination	No of mice in group	Avg CFU/ml of each dose
1	*L.lactis* LM2301 (Pip-CRR)	Nasal	20	5×10^{10}
2	*L.lactis* LM2301	Nasal	20	"
3	PBS	Nasal	20	
4	*L.lactis* LM2301 (Pip-CRR)	Subcutaneous	20	5×10^{10}
5	*L.lactis* LM2301	Subcutaneous	20	"
6	*L.lactis* LM2301 (Pip-CRR)	Nasal + Subcutaneous	19	"
7	*L.lactis* LM2301	Nasal + Subcutaneous	19	"
8	*L.lactis* LM2301 (Pip-CRR)	Nasal	18	2×10^{11}
9	*L.lactis* LM2301	Nasal	20	"
10	PBS	Nasal	20	

Comparison of Nasal and Subcutaneous immunization regimens. I first compared the immune responses of mice vaccinated with the same recombinant strains of *L.lactis* but by different routes of administration. Groups of 20 mice were vaccinated nasally, subcutaneously or combined with the recombinant *L.lactis* expressing the CRR of the M6 protein in the surface. Control mice were also vaccinated in a similar way with pip producing strains of *L.lactis* or PBS (Table 1).

Among the nasal immunized groups, all the mice that were given M6c expressing strain of *L.lactis*, group 1 (n=20) showed a S-IgA response in the saliva with a mean value of 0.88 ng/ml (Table 4). Only one mouse in the control group vaccinated with Pip expressing strain of *L.lactis*, group 2 (n=20), showed a small detectable value. In the control group 3 (n=20) which were given PBS, four mice showed a small response. The mean values of S-IgA in groups 2 and 3, 0.002 and 0.035 ng/ml respectively, were significantly lower than group 1 with P values of < 0.0001. The subcutaneous immunized mice did not have a significant IgA response in saliva in both the control and experimental groups. Seven mice in group 4 (n=20) that were injected with CRR expressing strain of *L.lactis* and two mice in group 5 (n=20) that were injected with Pip expressing strain of *L.lactis* showed a response (Figure 2). Statistical analysis of the mean responses in these two groups, 0.062. and 0.012 ng/ml did not show any significant difference.

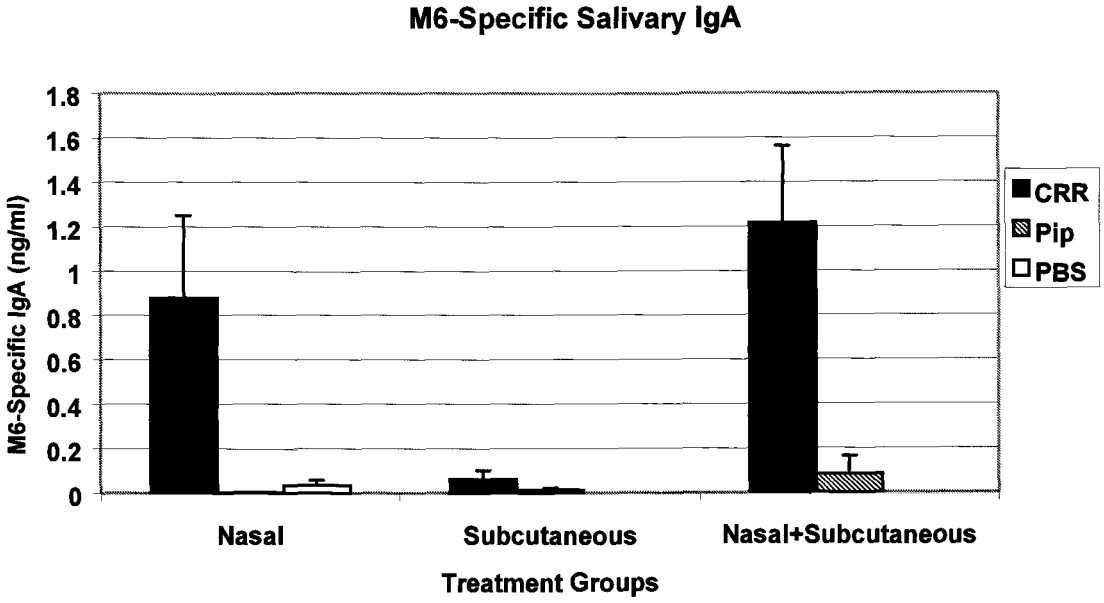

Figure 2. **M6 specific S-IgA levels.** Saliva IgA antibody responses in mice following immunization through various routes. Saliva was collected from mice 14 days after the last immunization and IgA antibody titres measured using a standard ELISA. The routes of immunization are given. Legend- CRR: Mice were immunized with *L.lactis* LM2301 (Pip-CRR), Pip: mice were immunized with *L.lactis* LM2301, PBS: Mice were immunized with PBS. Error bars indicate standard error.

The CRR specific IgG was measured in serum (Table 4). Seven mice in group 1 showed a response with a mean value of 19.98 ng/ml. Five mice in control group 2 showed response with a mean value of 6.96 ng/ml. The difference between these two groups was not statistically significant. Only one mouse in group 3 (n=20) showed a response. The mean response in group 3, 6.22 ng/ml, was not significant. Parenteral subcutaneous administration of recombinant *L.lactis* elicited a strong IgG response in serum. Sixteen mice in group 4 and six mice in control group 5 showed an increase (Figure 3). Statistical

analysis of the mean responses in these two groups, 57.41 and 7.19 ng/ml respectively showed a significant difference (P= 0.0010).

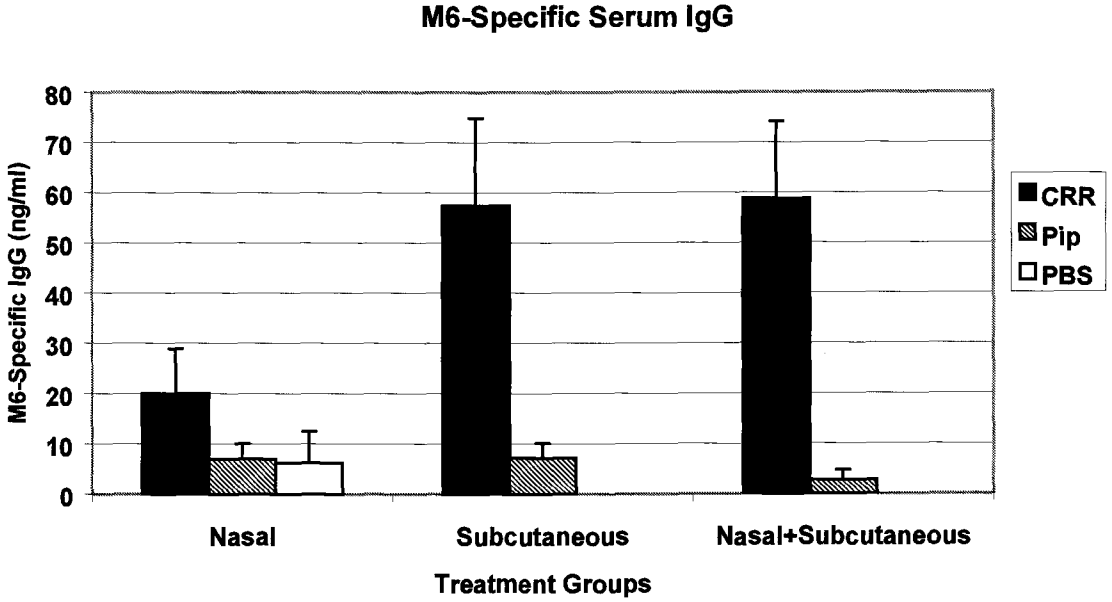

Figure 3. **M6 specific IgG levels.** Serum IgG antibody responses in mice following immunization through various routes. Serum was collected from mice 14 days after the last immunization and IgG antibody titres measured using a standard ELISA. The routes of immunization are given. Legend- CRR: Mice were immunized with *L.lactis* LM2301 (Pip-CRR), Pip: mice were immunized with *L.lactis* LM2301, PBS: Mice were immunized with PBS. Error bars indicate standard error.

Combination nasal and subcutaneous immunization regimen. I wanted to determine if combination immunization strategies could induce a more powerful systemic and mucosal immune response than either nasal or subcutaneous regimens alone (Table 4). All mice in the experimental group 6 (n=19) vaccinated with CRR expressing strain of *L.lactis* showed a significant increase (P <0.0001) in M6 specific S-IgA in saliva when compared to the control group 7 (n=19)

where one mouse showed a mean response of 0.086 ng/ml (Figure 2). The mean increase in the S-IgA in Group 6 was 1.21 ng/ml.

Similarly all mice in group 6 showed a strong post immune serum IgG response, with a mean response of 58.78 ng/ml while only two mice in group 7 showed a response in control Group 6 with a mean response of 2.77 ng/ml (Figure 3). This difference is significant ($P < 0.0001$).

Comparison of the results revealed that the combination regimen induced a higher antibody response, in both saliva and serum, than the individual regimens. The difference in the M6 specific S-IgA between groups 1 and 6 was significant ($P=0.0316$). However the difference in the M6 specific serum IgG between groups 4 and 6 was not significant.

Immunization with higher doses of *L.lactis* Mice were nasally vaccinated with four fold higher doses of recombinant *L.lactis* to determine if a better immune response and protection can be elicited (Table 4). The vaccination schedule used was similar to the previous experiments. All the mice that were given CRR expressing strain of *L.lactis*, group 8 (n=18) showed a S-IgA response in the saliva with a mean value of 0.74ng/ml. Three mouse in the control group vaccinated with Pip expressing strain of *L.lactis,* group 9 (n=20), showed a small detectable value. In the control group 10 (n=20) which were given PBS, three mice showed a small response (Figure 4).The mean values of S-IgA in groups 9 and 10, 0.003 and 0.064 ng/ml respectively, were significantly lower than group 8 with P values of < 0.0001.

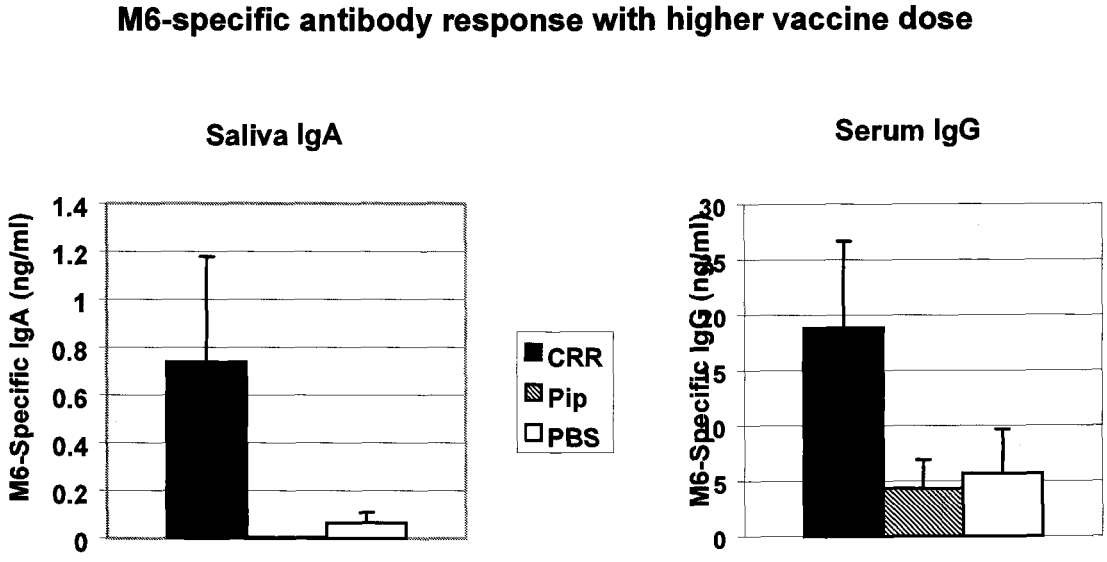

Figure 4. **Antibody response with higher vaccine dose.** The M6 specific saliva IgA and serum IgG response was tested in mice that were nasally immunized with four fold higher doses of recombinant *L.lactis*. Legend- CRR: Mice were immunized with *L.lactis* LM2301 (Pip-CRR), Pip: mice were immunized with *L.lactis* LM2301, PBS: Mice were immunized with PBS. Error bars indicate standard error.

The M6 specific IgG was measured in serum (Table 4). Six mice in group 8 showed a response with a mean value of 18.84 ng/ml. Three mice in control group 9 showed response with a mean value of 4.33 ng/ml. The difference between these two groups was not statistically significant. Only two mice in group 10 (n=20) showed a response. The mean response in group 10, 5.66 ng/ml, was not significantly lower than that seen in group 8 (Figure 4).

Comparison of the IgA and IgG responses between the two groups, group 1 and 8, given different nasal doses of recombinant *L.lactis* reveal that the responses was slightly lowered in the group given higher dose. These differences were not statistically significant.

Protection from infectious challenge. All the groups of mice, vaccinated, control vaccinated and PBS vaccinated, were challenged with equal nasal doses of *S.pyogenes*. Throat were swabbed for 11 days after challenge and streaked on blood agar plated with streptomycin to measure pharyngeal colonization. The results are shown on Table 2. The mice were scored as positive for infection if the swabs showed β hemolytic colonies from day 4 onwards or died after colonization (Table 3). All the dead mice except for one in group 5 showed positive throat cultures prior to death. This mouse was not considered in the following analysis.

Among the nasal vaccinated mice, five mice in group 1 were positive when compared to the control groups where fourteen were positive in group 2 and thirteen in group 3. This difference is statistically significant (P= 0.010 and 0.025 respectively). The subcutaneous vaccinated mice in group 4 were not afforded any significant protection when compared to its control group 5 (P=0.105). Nine mice were infected in group 4 as opposed to fifteen in group 5. Among the mice vaccinated with a combination regimen, group 6, four mice were infected (Figure 5). This was significantly less than control group 7, where thirteen mice were

infected (P= 0.0081). Six mice in group 8 were positive for colonization when compared to the control groups where fourteen were positive in group 9 and thirteen were positive in group 10. This difference is statistically significant (P= 0.0176 and P= 0.0437 respectively).

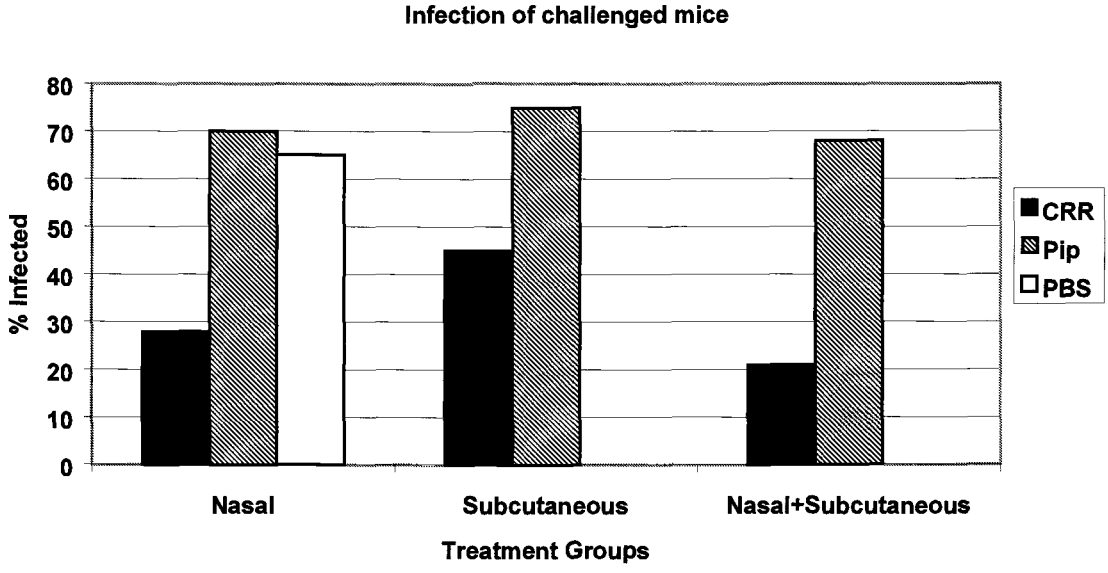

Figure 5. **Infection of challenged mice**. The various treatment groups were swabbed for pharyngeal colonization after they were challenged with nasal doses of *S.pyogenes*. The mice were followed for 11 days and scored as positive for infection if the swabs showed β hemolytic colonies from day 4 onwards or died after colonization. The routes of immunization are given. Legend- CRR: Mice were immunized with *L.lactis* LM2301 (Pip-CRR), Pip: mice were immunized with *L.lactis* LM2301, PBS: Mice were immunized with PBS

Comparison of the mortality of the challenged mice shows that there were no deaths in any of the groups vaccinated with M6 expressing strain of *L.lactis*, i.e Groups 1,4,6 (Table 3). In contrast the control groups show mortality of 11/20 (55%) in Group 2, 10/20 (50%) in Group 3, 11/20 (55%) in Group 5 and 6/19

(32%) in Group 7. Fisher's exact test revealed that the differences in mortality rates between the mice vaccinated with recombinant *L.lactis* and the control groups were highly significant (P <. 001). There were no significant differences in the mortality of the different control mice. One mouse in group 8 died during the challenge study. In the control groups 9 and 10, nine and twelve mice died after challenge (Figure 6). The difference in mortality between group 8 and the control groups 9 and 10 was statistically significant (P <0. 001)

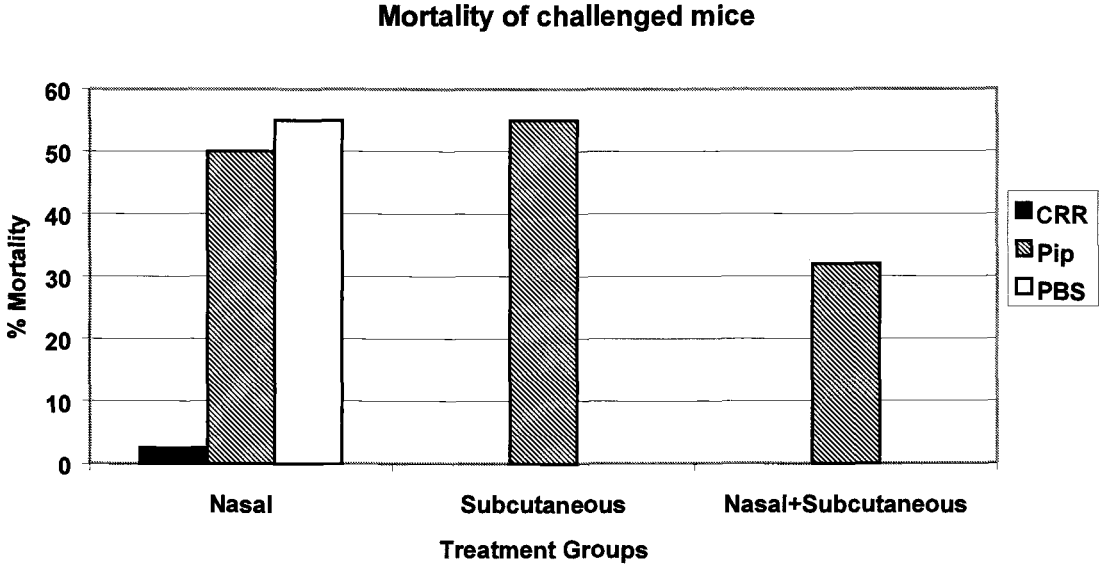

Figure 6. **Mortality of challenged mice**. The various treatment groups challenged with nasal doses of *S.pyogenes*. The mice were followed for 11 days after challenge. The routes of immunization are given. Legend- CRR: Mice were immunized with *L.lactis* LM2301 (Pip-CRR), Pip: mice were immunized with *L.lactis* LM2301, PBS: Mice were immunized with PBS

Correlation between immune response and protection The results indicate a broad relationship between the antibody response and the level of protection in different groups of mice. To get a better idea of this correlation I took a closer look at the data for mice in all the groups. All 195 mice in the study, in experimental and control groups, were first separated into infected and non-infected groups (Table2, Figure 7). The criteria for considering a mouse positive for infection are explained earlier. The difference between the mean S-IgA and serum IgG between these two groups was significant (P< 0.0001 and P=0.0130 respectively).

Table 2. **Correlation of antibody response and protection.** All the mice in the study were grouped using infection, mortality or mortality after pharyngeal colonization as criteria. The mean S-IgA and serum IgG values were calculated and compared. P values are indicated beside each group.

	Saliva IgA ng/ml	P value	Serum IgG ng/ml	P value
Infected	.09115	<0.0001	11.352	0.0130
Non- Infected	0.5695		28.436	
Mice that died during challenge	0.04129	<0.0001	5.987	0.0018
Mice that survived challenge	0.4197		24.480	
Colonization	0.1591	0.0008	17.248	0.0205
Colonization + Death	0.04101		4.974	

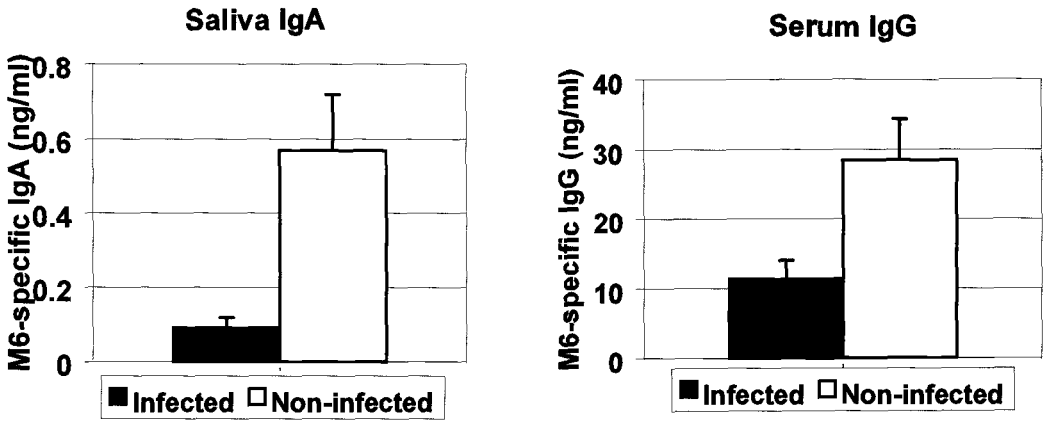

Figure 7. **Correlation of antibody response and infection.** M6 specific saliva IgA and serum IgG was compared between infected and non-infected mice. Error bars indicate standard error.

All the experimental and control mice were also grouped using mortality as the sole criteria. This was done to determine the contribution of the local mucosal immunity and systemic immunity to protection against death. The M specific antibody levels antibody levels were compared between mice that died and mice that remained alive after challenge with *S.pyogenes* (Table 2, Figure 8). Included in the live group are mice that survived for 11 days after challenge despite pharyngeal colonization. The difference between the mean S-IgA and serum IgG between these two groups was significant ($P < 0.0001$ and $P=0.0018$ respectively).

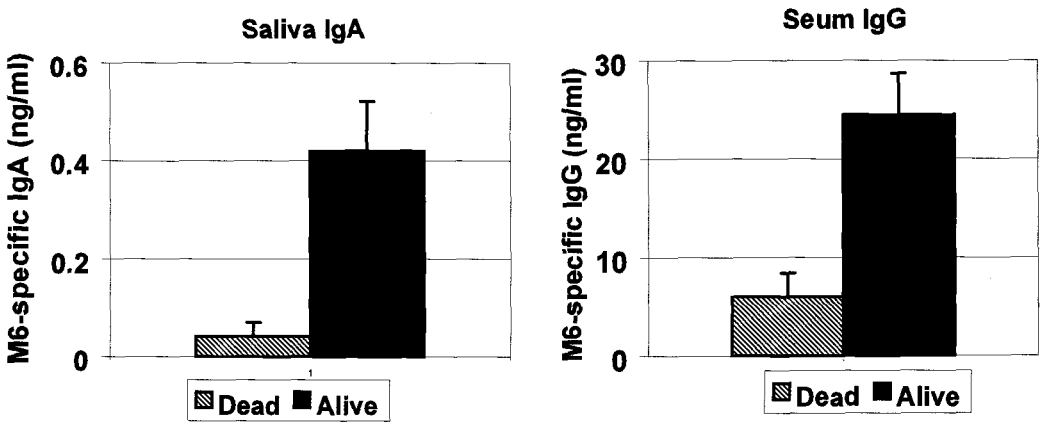

Figure 8. **Correlation of antibody response and mortality.** M6 specific saliva IgA and serum IgG was compared between mice that died and mice that remained alive after challenge with *S.pyogenes*.. Error bars indicate standard error.

105 mice in all the groups showed pharyngeal colonization and 65 among them died during challenge. The colonized mice were separated according to mortality and the antibody responses were compared (Table 2, Figure 9). This was done to determine the contribution of serum IgG to survival of challenged mice. The difference between the mean S-IgA and serum IgG between these two groups was significant (P=0.0008 and P=0.0205 respectively).

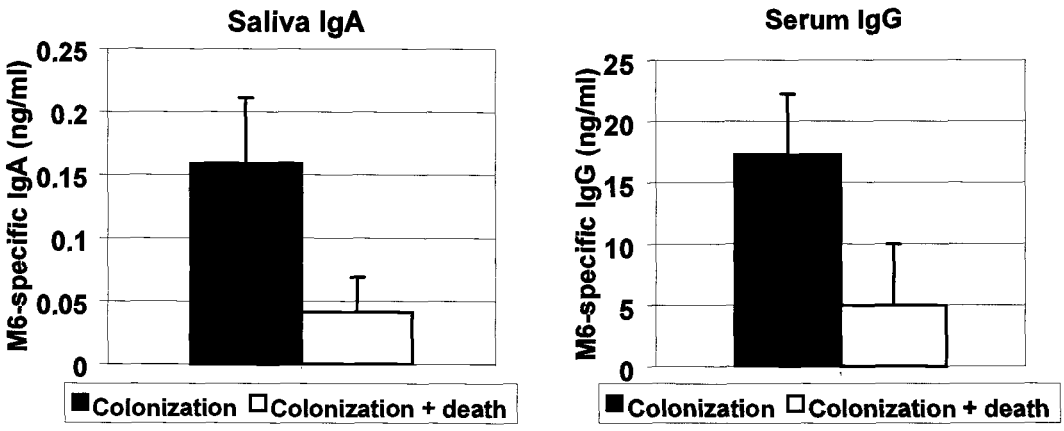

Figure 9. **Correlation of antibody response and mortality among colonized mice.** M6 specific saliva IgA and serum IgG was compared between colonized mice that died and colonized mice that remained alive after challenge with *S.pyogenes*. Error bars indicate standard error.

To test the hypothesis that serum IgG has no role in prevention of colonization I examined the antibody results of subcutaneous vaccinated mice in group 4. These mice had high levels of M6c specific serum IgG antibodies but no IgA levels in saliva. Nine mice in this group were positive for pharyngeal colonization of *S.pyogenes*. The mean IgG value in the colonized mice was 46.06 ng/ml. The rest of the mice in the group had an average IgG response of 75.37 ng/ml (Figure 10). Although the value was higher, statistical analysis did not show a significant difference ($P = 0.4411$) indicating that M6 specific serum IgG does not contribute to protection against mucosal infection.

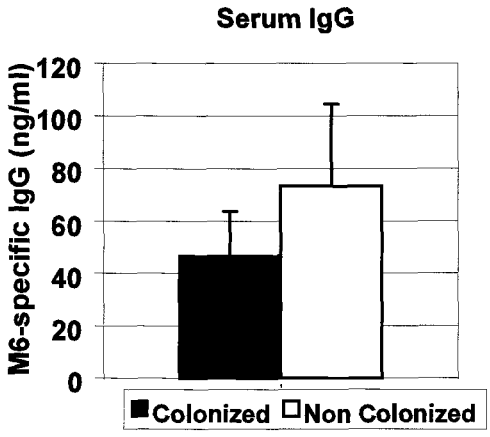

Figure 10.**Serum IgG among colonized and non-colonized mice in subcutaneous group.** M6 specific IgG was compared among the colonized and non-colonized mice in the subcutaneous vaccinated group. Error bars indicate standard error.

Table 3. **Pharyngeal colonization and death of challenged mice**. Groups of vaccinated mice were swabbed for 11 days colonization following challenge with nasal dose of *S.pyogenes*. +: Colonization, -: No colonization, d: Death.

Mouse No	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Group1																				
Day 4	-	-	-	-	+	-	-	+	-	+	-	-	-	-	-	-	-	+	-	+
Day 5	-	-	-	-	+	-	-	+	-	+	-	-	-	-	-	-	-	+	-	+
Day 7	-	-	-	-	+	-	-	+	-	+	-	-	-	-	-	-	-	+	-	+
Day 9	-	-	-	-	+	-	-	+	-	+	-	-	-	-	-	-	-	+	-	+
Day 11	-	-	-	-	+	-	-	+	-	+	-	-	-	-	-	-	-	+	-	+
Group2																				
Day 4	+	-	+	+	-	+	-	+	+	+	-	+	+	-	-	+	-	+	+d	-
Day 5	+	-	+d	+	-	-	-	+	+	+	-	+d	+d	-	-	+	-	+d		-
Day 7	+	-		+	-	-	-	+	+	+	-	-		-	-	+	-			-
Day 9	+d	-		+d	-	+d	+d	+d	+	+	-	-		-	-	+	+d			-
Day 11		-			-				+	+	-	-		-	-					
Group3																				
Day 4	-	-	+	+	-	-	-	+	-	+	-	-	-	-	+	-	-	+	-	+
Day 5	+	-	+	+	-	-	-	+	-	+	-	-	+	+	+	-	+	+	-	+d
Day 7	+	+	+d	+	-	-	-	+	-	-	+	-	+	+	+d	-	+	+d	-	
Day 9	+	-		+	-	-	-	+d	-	-	+d	-	+d	+d		-	+d		-	
Day 11	+	-		+	-	-	-		-	+d		-				-			-	
Group4																				
Day 4	-	-	-	-	-	+	-	+	+	+	-	-	-	+	-	-	-	-	-	-
Day 5	-	-	-	-	-	+	-	+	+	+	-	+	+	+	-	-	-	-	-	-
Day 7	-	-	-	-	-	+	-	+	+	+	+	+	+	+	+	-	-	-	-	-
Day 9	-	-	-	-	-	+	-	+	+	+	+	-	+	+	+	-	-	-	-	-
Day 11	-	-	-	-	-	+	-	+	+	+	+	-	+	+	+	-	-	-	-	-
Group5																				
Day 4	+	+	+	+	+d	-	-	-	-	-	-	-	+	-	-	-	-	-	+	+
Day 5	+d	-	+	+		-	+	+	-	+	-	-	+	-	-	-	-	d	+	+
Day 7		+	+	+d		-	+	+	-	+	-	-	+	-	+	+	-		+d	+
Day 9		+	+			+d	+	+d	+d	+d	-	-	+	-	+d	+d	-			+d
Day 11		+	+				+				-	-	+	-			-			
Group6																				
Day 4	-	-	-	+	+	-	-	-	+	-	-	+	-	-	-	-	-	-	-	
Day 5	-	-	-	+	+	-	-	-	+	-	-	+	-	-	-	-	-	-	-	
Day 7	-	-	-	+	+	-	-	-	+	-	-	+	-	-	-	-	-	-	-	
Day 9	-	-	-	+	+	-	-	-	+	-	-	+	-	-	-	-	-	-	-	
Day 11	-	-	-	+	+	-	-	-	+	-	-	+	-	-	-	-	-	-	-	
Group7																				
Day 4	-	-	+	+	-	-	-	+	+	-	+	+	-	+	+	+	-	+		
Day 5	-	-	+	+	-	-	-	-	+	+	+	+	-	+	+	+	-	+d		
Day 7	-	-	+	+	+	-	-	-	+	-	+	+	-	+	+	+	-			
Day 9	-	-	+	+	+d	-	-	-	+	-	+d	+	-	+d	+	+d	-			
Day 11	-	+	-	+		-	-	+d	+	-		+	-		+		-			

Table 3. Continued

Mouse no	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Group8																				
Day 4	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	+	-		
Day 5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+d	-	+	-		
Day 7	-	-	-	-	-	+	-	-	+	-	-	-	-	-		-	+	-		
Day 9	-	-	-	-	-	+	-	-	+	-	-	-	-	-		+	+	-		
Day 11	-	-	-	-	-	+	-	-	+	-	-	-	-	-		+	+	-		
Group9																				
Day 4	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+		
Day 5	-	+	-	-	-	+d	-	-	-	-	-	-	-	+	-	+	-	+	+	-
Day 7	+	+	+	-	-		-	-	-	+d	-	-	-	+	+	+d	+d	+d	d	-
Day 9	+	+	+d	-	-		+d	--	-		+	-	-	+	+d					-
Day 11	+	+		-	-			-	-		+	+	-	+						-
Group10																				
Day 4	-	-	-	+	-	-	-	-	-	+	-	-	+d	-	-	-	-	+	-	-
Day 5	-	+	-	+	-	-	-	+d	-	+	-	-		-	-	-	-	+	+	+
Day 7	-	+	-	+	-	-	-		+	+	-	-		-	-	+d	-	+d	+	-
Day 9	-	+	+	+d	-	+d	-		+	+d	-	-		-	+		-		+d	+d
Day 11	-	+d	+d		-		-				-	-		-	+d		-			

Table 4. **S-IgA and serum IgG response in treatment groups.** The M6c specific IgA antibodies in saliva and IgG antibodies in serum was measured after vaccination using standard ELISA.

	Group1 (Pip emmp16) Nasal route		**Group 2 (Pip wtp16) Nasal route**		**Group 3 (PBS) Nasal route**	
Mouse no	Saliva IgA ng/ml	Serum IgG ng/ml	Saliva IgA ng/ml	Serum IgG ng/ml	Saliva IgA ng/ml	Serum IgG ng/ml
1	0.192902	0	0	0	0.013159	0
2	2.824513	0	0	0	0	0
3	0.048508	141.6969	0	17.77778	0	0
4	7.05223	31.28684	0	0	0.065567	0
5	0.303495	0	0	0	0	0
6	0.514216	0	0	0	0	0
7	0.408856	0	0	31.27301	0	0
8	0.699906	0	0	0	0	0
9	0.186538	0	0	0	0	0
10	0.412957	0	0	34.35631	0.1599	0
11	2.93652	20.88295	0	0	0	124.3443
12	0.427382	6.155887	.002263	0	0	0
13	0.098289	0	0	0	0.451393	0
14	0.468961	0	0	0	0	0
15	0.167304	0	0	0	0	0
16	0.241269	31.97209	0	0	0	0
17	0.16985	0	0	10.32979	0	0
18	0.041296	111.0598	0	0	0	0
19	0.137747	0	0	45.47697	0	0
20	0.226702	56.55259	0	0	0	0
Mean Values	0.87797205	19.980353	0.0022628	6.960693	0.034501	6.217215

Table 4. Continued

Mouse No	Group 4 (Pip emmp16) Subcutaneous route		Group 5 (Pip wt16) Subcutaneous route	
	Saliva IgA ng/ml	Serum IgG ng/ml	Saliva IgA ng/ml	Serum IgG ng/ml
1	0	5.02603	0	40.37846
2	0	86.95578	0	0
3	0	54.75389	0.079077	27.3169
4	0.029821	54.24784	0	0
5	0	178.7808	0.163557	12.04485
6	0	0	0	0
7	0.017313	65.83852	0	0
8	0	25.71734	0	25.06426
9	0.023461	7.545688	0	0
10	0	21.20402	0	34.84949
11	0	165.1742	0	0
12	0.192989	51.87552	0	0
13	0.129178	33.07042	0	0
14	0	23.00015	0	0
15	0	0	0	0
16	0	21.16027	0	0
17	0	311.069	0	4.163195
18	0.811105	0	0	0
19	0	0	0	0
20	0.029856	42.87302	0	0
Mean Values	0.06168615	57.414624	0.0121317	7.1908578

Table 4. Continued

Mouse No	Group 6 (Pip emmp16) Nasal +Subcutaneous route		Group 7 (Pip wt16) Nasal+Subcutaneous route	
	Saliva IgA ng/ml	Serum IgG ng/ml	Saliva IgA ng/ml	Serum IgG ng/ml
1	1.165705	27.02655	0	0
2	1.379693	5.924575	0	0
3	0.730652	53.14307	0	0
4	1.344749	19.66591	0	0
5	1.134747	0.766546	1.646542	0
6	6.331745	266.9664	0	16.83532
7	0.608697	0.537969	0	0
8	0.245135	107.2044	0	0
9	0.125411	37.50331	0	0
10	0.4267	87.32253	0	35.84696
11	0.375081	16.70197	0	0
12	0.919201	34.57073	0	0
13	0.155	55.93957	0	0
14	1.039788	22.33726	0	0
15	0.535265	35.43485	0	0
16	0.173646	48.26606	0	0
17	1.540425	189.7031	0	0
18	0.438291	45.78163	0	0
19	4.425742	62.04472	0	0
Mean Values	1.21556174	58.781113	0.0866601	2.7727516

Table 4. Continued

Mouse no	Group8 (Pip emmp16) Nasal route		Group 9 (Pip wtp16) Nasal route		Group 10 (PBS) Nasal route	
	Saliva IgA ng/ml	Serum IgG ng/ml	Saliva IgA ng/ml	Serum IgG ng/ml	Saliva IgA ng/ml	Serum IgG ng/ml
1	7.895823	110.45667	0.01188	0	0	67.56767
2	0.230096	0	0	0	0	0
3	0.108189	0	0	0	0	0
4	0.106209	0	0	45.45766	0.04568	0
5	0.224581	44.56788	0	0	0.427807	0
6	1.289076	60.5467	0	0	0	0
7	0.218782	0	0	0	0.80866	0
8	0.107341	38.9657	0	0	0	0
9	0.079339	0	0	0	0	0
10	0.123039	0	0	17.6588	0	0
11	0.133362	0	0.05063	0	0	0
12	0.257532	0	0	0	0	0
13	2.148791	10.23455	0	23.56757	0	0
14	0.073682	0	0.008203	0	0	45.67899
15	0.00792	0	0	0	0	0
16	0.032103	0	0	0	0	0
17	0.001414	0	0	0	0	0
18	0.2711090	74.4578	0	0	0	0
19			0	0	0	0
20			0	0	0	0
Mean Values	0.739355	18.84607	0.003536	4.334201	0.0641074	5.662333

DISCUSSION

A comparison of the immune response with three different regimens of vaccination showed that nasal delivery of the vaccine strain of *L.lactis* was necessary for production of local immunity at mucosa. CRR-specific IgA response in saliva was seen only with nasal vaccination and not with subcutaneous vaccination. It has been recognized that the mucosal immune system is a distinct part of the immune apparatus and functions independently of the systemic immune system (44). S-IgA, the principal antibody of the mucosal membrane, is produced locally by large number of activated plasma cells in the subepithelial spaces of mucosa and in the secretory glands. There is no significant entry of systemic derived IgA into the mucosal secretions. Parenteral administration of antigens generates a strong systemic immune response with no corresponding mucosal response (46). Thus a reliable and consistent method of producing a specific immune response at mucosal sites is through mucosal application of the antigen.

A combination of subcutaneous vaccination followed by nasal vaccination was examined to determine if former would "prime" the latter and lead to an elevated level of CRR-specific salivary IgA. In this study CRR specific S-IgA levels after the combination regimen were significantly higher than the nasal regimen.

Subcutaneous vaccination produced a three-fold higher level of CRR-specific serum IgG than nasal vaccination. The serum IgG response from the combination regimen was about the same as subcutaneous alone. The data support the study done by Aase A et al (1)

Pharyngeal colonization following challenge with *S.pyogenes* was significantly reduced in vaccinated mice that presented CRR-specific salivary S-IgA. The mice that received nasal doses of recombinant *L.lactis*, either alone or in combination with subcutaneous doses, were protected against colonization. The group that received only subcutaneous vaccination was not protected against colonization despite high levels of CRR-specific IgG in serum. In this group the mean IgG levels between the colonized and non-colonized mice was not significantly different. This indicates that protection against colonization of *S.pyogenes* does not involve CRR-specific IgG in serum. This is perhaps not surprising, considering that adhesion to pharyngeal epithelium is mediated by M protein (14). Mucosal antibodies against CRR of M protein and other surface adhesins protect against colonization with group A streptococci by inhibiting adhesion (14). Passive administration of M protein specific IgA protected against mucosal GAS infection in mice (5). In vitro studies show that secretory IgA specific to M protein prevented attachment of group A streptococci to pharyngeal epithelial cells. M protein specific IgG did not prevent attachment (25). Thus mucosal immunity is important in protection against pharyngeal colonization by GAS.

Survival of immunized mice after nasal challenge with S.pyogenes correlated with the presence of both CRR-specific S-IgA and serum IgG. Among all the groups vaccinated with recombinant *L.lactis* only one out of twenty-four mice with pharyngeal colonization died. In the control groups fifty-nine mice out of eighty-two died after challenge. The CRR specific S-IgA and serum IgG levels in mice that ultimately died were significantly lower than in surviving mice. The contribution of CRR-specific serum IgG to survival is evident on examination of the subcutaneous vaccinated mice. None of the nine mice with pharyngeal colonization died after challenge in spite of the absence of an IgA response in saliva. In contrast eleven out of fifteen colonized mice in the subcutaneous control group died. Despite the lower levels of CRR-specific serum IgG in nasally immunized mice compared to those vaccinated subcutaneously, protection from the lethal effect of (presumably) systemic invasion was the same as that in mice that were vaccinated subcutaneously.

In *S.pyogenes* infections the initial adhesion and colonization is followed by invasion of pharyngeal epithelium and systemic dissemination leading to death (14). S-IgA, by blocking adhesion, inhibits this process at the first step itself (5). Interestingly, among the colonized mice in this study, the levels of CRR-specific saliva IgA in mice that died were significantly lower than in mice that survived challenge. This suggests that even after colonization S-IgA can reduce mortality, probably by lowering the bacterial load in the pharynx. Anti-M IgG binds to surface of streptococci and facilitates clearance by opsonization and is

important in protection against streptococci that have invaded the host tissue (14). M-specific IgG did not prevent adhesion but reduced invasion of group A streptococci into pharyngeal epithelial cells in vitro (25). Thus protective immunity against GAS infections involves two complimentary modes of protection mediated by S-IgA and IgG (14). This concept is supported by the findings in this study.

Immune response to the conserved C region of M protein is important for protection against GAS infections (53). The conserved regions of the M protein including the C region are often not immunogenic or evoke a low level of antibodies (53,17). The vaccine constructs based on the conserved C region were effective in animal trials. However these studies required the use of mucosal adjuvants, such as cholera toxin subunit B or diptheria toxoid, along with the C region peptides for an effective immune response (4,6,51). Vaccinia virus that expresses the conserved regions of M protein has been shown to protect against streptococcal pharyngeal colonization (23). Medical concerns about the toxicity of the adjuvants and safety of vaccinia-based vaccines preclude their use in humans. Here I show that CRR of M protein that is displayed in the context of immunoadjuvant effect of *L.lactis* is effective against streptococcal infection. My results support the concept that *L.lactis*, which is consumed regularly in dairy products, is a safe and effective vaccine vector for human use.

The high copy plasmid pBG568 used in this study has erythromycin resistance gene for selection. The use of this selection marker is not advisable

for administration to human subjects because of concern of transfer of antibiotic resistance genes to other bacteria in the GIT. Placement of the Pip-M6c gene on the chromosome would probably decrease expression of CRR. Alternative plasmid selection markers such as β-Gal or nissin, might be used to replace the erythromycin resistance gene so that plasmid-bearing strains of the vaccine could be used safely in humans. The immunogenicity of the current vaccine construct might be increased by using tandem repeats of CRR or a stronger promoter to increase the levels of antigen displayed on the surface. Multivalent M protein constructs containing epitopes from type specific regions of different M proteins might also be investigated.

The data presented in this study brings us closer to the development of a safe, effective mucosal vaccine against *S.pyogenes* and also open the potential of using the pip protein to express other heterologous proteins on the surface of *L.lactis* with the aim of using them as vaccines.

CONCLUSIONS

- Immunization of mice with recombinant *L.lactis* expressing the C repeat region (CRR) of M6 protein stimulated an antigen specific antibody response in saliva and serum, which reduced the colonization and death of mice following nasal challenge with *S.pyogenes.*
- Nasal vaccination is necessary for development of mucosal immunity.
- Mucosal immunity is required for prevention of colonization of *S. pyogenes.*
- Subcutaneous vaccination is not effective in producing a mucosal immune response.
- Subcutaneous vaccination produces a stronger serum IgG response than nasal vaccination.
- The immune response and protection from a combination regimen of nasal plus subcutaneous vaccination is higher than that seen with only nasal vaccination.
- There is an inverse correlation between CRR-specific serum IgG levels and death.
- Use of *L.lactis* as a vaccine vector eliminated the need for adjuvants to render CRR antigenic.

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