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Mucosal and systemic immune responses to a recombinant protein expressed on the surface of the oral commensal bacterium *Streptococcus gordonii* after oral colonization

(secretory IgA/oral vaccine/vaccine vector/M protein/Gram positive)

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ABSTRACT **To circumvent the need to engineer pathogenic microorganisms as live vaccine-delivery vehicles, a system was developed which allowed for the stable expression of a wide range of protein antigens on the surface of Gram-positive commensal bacteria. The human oral commensal *Streptococcus gordonii* was engineered to surface express a 204-amino acid allergen from hornet venom (Ag5.2) as a fusion with the anchor region of the M6 protein of *Streptococcus pyogenes*. The immunogenicity of the M6–Ag5.2 fusion protein was assessed in mice inoculated orally and intranasally with a single dose of recombinant bacteria, resulting in the colonization of the oral/pharyngeal mucosa for 10–11 weeks. A significant increase of Ag5.2-specific IgA with relation to the total IgA was detected in saliva and lung lavages when compared with mice colonized with wild-type *S. gordonii*. A systemic IgG response to Ag5.2 was also induced after oral colonization. Thus, recombinant Gram-positive commensal bacteria may be a safe and effective way of inducing a local and systemic immune response.**

Mucosal surfaces, where the vast majority of infections begin, constitute the first barrier encountered by microbial pathogens (1). The specific immunological defense at this site is primarily mediated by antibodies of the immunoglobulin A class (IgA) (2, 3). Several examples exist in which resistance to infection may be directly correlated with the presence of antigen-specific IgA (1, 4, 5). While there is evidence of a common mucosal immune system (6), mounting data indicate that the presentation of antigen at a specific mucosal site results in higher levels of IgA at that site when compared with distant mucosal locations (7, 8).

Live microbial vectors that actively multiply at mucosal surfaces are more efficient than killed vectors at stimulating a secretory response to a recombinant antigen (reviewed in ref. 9). However, in most instances, these live antigen-delivery vectors comprise bacteria or viruses that are normally mammalian pathogens engineered to reduce their pathogenicity, yet maintain certain invasive/adherence qualities to induce an immune response (10, 11). To circumvent some of the safety and environmental issues inherent in the wide-scale dissemination of engineered pathogens, we have developed a system whereby nonpathogenic Gram-positive commensal bacteria that occupy a specific mucosal niche may be used to stimulate a mucosal immune response against a pathogen that enters the mammalian host at a specific site (oral, intestinal, or vaginal) (12).

In our model system, we genetically removed nearly all of the surface exposed region of the M6 protein of *Streptococcus pyogenes* and replaced it with a foreign antigen, retaining the C-terminal attachment motif of the M6 protein (13, 14). The strain Challis of *Streptococcus gordonii*, a human oral commensal bacterium, was chosen as the vector organism. As proof of the general applicability of the system, Ag5.2, a 204-amino acid allergen of the white-face hornet venom (15), was expressed on the surface of *S. gordonii*. The mucosal IgA response in different secretions (saliva, lung, and intestinal fluids) together with the systemic IgG response were assayed in mice colonized with the recombinant organisms after a single oral/intranasal inoculum.

MATERIALS AND METHODS

Construction of Recombinant *S. gordonii*. Insertion vector pSMB55 (M. R. Oggioni and G.P., unpublished data) is a 5.73-kb *Escherichia coli* plasmid that does not replicate in streptococcal strains but is capable of integrating in the chromosome of the recipient *S. gordonii* strain GP251 (16) by recognition of flanking sequences.

Plasmid pSMB55, a derivative of pVMB20 (12), carries a 2.9-kb fragment containing the M6 gene (*emm6.1*) (17), in which the 538-bp *Kpn* I–*Hin*dIII fragment that codes for the central portion of the cell surface exposed region of the M6 molecule (18) was deleted and substituted with a 27-bp polylinker. Translational gene fusions with the *emm6.1* gene can be obtained by cloning in pSMB55. Procedures for cloning, gene fusions, transformation of *S. gordonii*, scoring, and genetic analysis of transformants were as described (12, 16, 19, 20).

Ag5.2-Encoding DNA. To clone the 616-bp DNA encoding Ag5.2 in the *S. gordonii* expression system, we amplified the cloned *f10* sequence (15) by PCR. The sense primer (position 1–18; ref. 15) CTGGGATCCAATAATTATTGTAAGATA contains a *Bam*HI restriction site and the antisense primer (position 598–615; ref. 15) CGTAGATCTTTTTCTTTCATAAATTGG contains a *Bgl* II restriction site.

Ag5.2 Preparation. Recombinant and native Ag5.02 were isolated and purified from *Escherichia coli* and the white-face hornet venom, respectively, as described (35) and referred to here as Ag5.2.

Immunofluorescence. Immunofluorescent staining of streptococcal cells was performed as described (16).

Western Blot Analysis of Cell Fractions. Streptococcal cells were separated into cell wall extract and cytoplasmic and membrane fractions and Western blotted as described (16).

Bacterial Growth and Immunization of Mice. Six-week-old female BALB/c mice were obtained from The Jackson Laboratory. Baseline serum and saliva samples were collected.

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S. gordonii wild-type (GP204) and recombinant (DM100) strains were used for colonization and immunization studies. Both GP204 and DM100 strains contain a mutant allele, str-204, conferring resistance to high levels (>1 mg/ml) of streptomycin. Both strains were grown in tryptic soy broth (Difco) containing streptomycin (500 μg/ml). Ten milliliters of the bacterial culture, grown at 37°C until the end of exponential phase (OD_{590} = 1.2), was centrifuged, and the bacterial pellet was washed once with saline and resuspended in fresh medium (1/20th of the original volume).

For 2 days prior to inoculation, mice were supplied with drinking water containing 5 g of streptomycin per liter. Mice received a single oral/intranasal inoculum with a total of 50 μl of the bacterial suspension (≈8×10^8 colony-forming units) of which 10 μl was delivered to each nostril and 30 μl to the mouth by using a micropipette. Ten mice were inoculated orally and intranasally with live, wild-type strain GP204, 23 with live, recombinant DM100 and 6 with mitomycin-killed (21), recombinant DM100. Ten mice were immunized subcutaneously with the same amount of the recombinant DM100 cells emulsified in complete Freund's adjuvant.

Colonization Analysis. Oral/pharyngeal swabs of orally/intranasally immunized animals were taken weekly (Calgiswab type 4) and cultured on blood-agar plates containing streptomycin (500 μg/ml). Positive colonies were picked with sterile tooth picks and tested for erythromycin resistance on plates containing erythromycin (1 μg/ml) and expression of M6 protein by the colony immunoblotting technique (19). Swabs displaying more than two colonies resistant to both streptomycin and erythromycin and expressing M6 protein were considered positive for the presence of recombinant *S. gordonii*.

To determine the sites colonized by *S. gordonii* after a single oral/intranasal inoculation, mice (n = 19) were sacrificed at different time points and aseptically dissected, and samples were collected from specific areas of the respiratory and gastrointestinal tracts (nose, gums, teeth, tongue, hard palate, perisinus, esophagus, trachea, lung, stomach, small intestine, colon, and cecum). All samples were tested for the presence of wild-type and recombinant *S. gordonii* as described above.

Sample Collection. Blood samples were collected from the retroorbital plexus of anesthetized mice 0, 4, 7, and 11 weeks after immunization, and serum was stored at −70°C. Saliva was collected after stimulation with pilocarpine (5 mg per kg of body weight, injected s.c.), centrifuged at 15,000 × *g* at 4°C for 20 min, and the supernatant stored at −70°C. After 11 weeks, mice were sacrificed, and their lungs were excised and washed three times by injecting 0.5 ml of ice-cold saline (0.85% NaCl) into the trachea. Samples were centrifuged at 2500 × *g* for 20 min at 4°C and stored at −70°C. The small intestine was removed from the same animals, the lumen was washed three times with 0.5 ml of ice-cold saline, and samples were centrifuged at 10,000 × *g* for 20 min at 4°C. Bovine serum albumin (0.01%) was added to intestinal sample before they were stored at −70°C. Phenylmethylsulfonyl fluoride (1 mM) and sodium azide (0.01%) were added to saliva and lung and intestinal lavage immediately after collection.

ELISA. Anti-Ag5.2-specific secretory IgA in saliva and lung and intestinal lavages was quantitated by ELISA. Microtiter plates were sensitized with 5 μg of Ag5.2 per ml, as described (22). The total IgA in samples was determined by using plates coated with 1 μg of goat anti-mouse IgA per ml (Southern Biotechnology Associates). The IgA concentration in each sample was calculated against a standard curve of mouse myeloma IgA (Cappel) determined simultaneously in the same plate. For antigen-specific IgA, saliva and intestinal lavage samples were diluted 1:1, while lung lavage was diluted 1:5 in blocking buffer (22), containing 1% bovine serum albumin.

To determine total IgA concentration in the various fluids, saliva samples were initially diluted 1:20, while intestinal and lung washes were diluted 1:50, and titrated by 1:1 dilutions. After incubating overnight at 4°C, alkaline phosphatase-conjugated goat anti-mouse IgA (Kirkegaard & Perry Laboratories) was added at a dilution of 1:1000. Plates were incubated at 37°C for 3 h, washed, substrate was added (22), and plates were read after 2-h incubation at room temperature at 405 nm by using a Dynatech MR4000 plate reader. Results are expressed as the percentage of Ag5.2-specific IgA in relation to the total IgA.

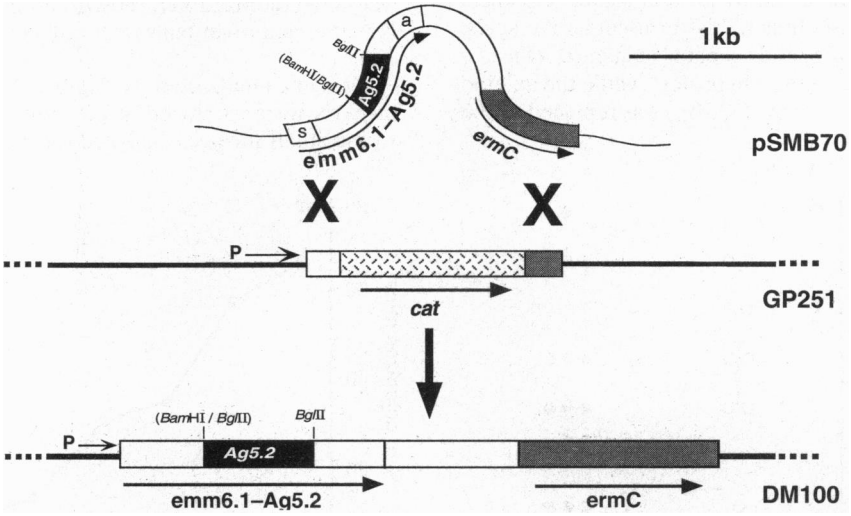

FIG. 1. Integration of the *emm6.1*–Ag5.2 translational fusion in the chromosome of *S. gordonii*. In recombinant plasmid pSMB70, the Ag5.2 gene (616 bp; in black) is inserted in frame within the M6 protein gene (*emm6.1*) replacing the central region (538 bp) of *emm6.1*. The open reading frame of *emm6.1*–Ag5.2 gene fusion and *ermC* are marked by arrows. Regions of *emm6.1* encoding the signal peptide (s) and the C-terminal anchor (a) of the M6 protein are indicated. Recipient strain GP251 is engineered such that a promoterless chloramphenicol acetyltransferase (*cat*) gene (broken cross-hatched lines) flanked by two short DNA segments (also present in plasmid pSMB70) is located downstream from a strong chromosomal promoter (P). The short 5′ segment (white) contains the first 145 nt of the *emm6.1* gene, and the short 3′ segment (grey), the last 200 nt of the *ermC* gene. pSMB70 was used as donor in the transformation of recipient strain GP251. The flanking homologous segments allowed for the integration of the M6–Ag5.2 gene fusion together with the *ermC* gene into the chromosome of GP251. Transformants were selected for erythromycin resistance and tested for the expression of the M6–Ag5.2 protein. DM100 is a representative transformant.

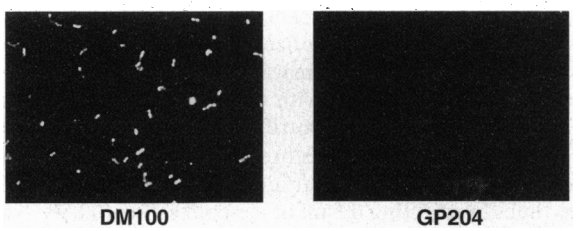

FIG. 2. Immunofluorescence of recombinant *S. gordonii*. Recombinant DM100 was treated with Ag5.2-specific monoclonal antibody and anti-mouse antiserum conjugated with fluorescein isothiocyanate. DM100 showed fluorescent staining, whereas wild-type GP204 was negative. Both micrographs were exposed for the same length of time. (×1000.)

Kinetic ELISA was used to determine serum IgG titers (22). Plates were coated as described above. Serum samples were diluted 1:20 in blocking buffer containing 1% bovine serum albumin. After the reaction had proceeded for 3 h at 37°C, the plate was washed and a 1:1000 dilution of goat anti-mouse IgG (Sigma) [adsorbed 1:1 (vol/vol) with packed DM100 cells] was added. Plates were read for 1 h at 4-min intervals on an ELIDA-5 plate reader (Physica, New York) (22).

Data Analysis. Samples from three separate experiments comprising 49 animals were tested individually. Results are expressed as the mean ± SEM. Statistical significance was determined by Student's unpaired *t* test. Simple linear regression and calculation of Pearson's correlation coefficient were performed. The significance level was set at $P < 0.05$.

RESULTS

Construction and Characterization of Recombinant *S. gordonii*

M6–Ag5.2 Gene Fusion. The 616-bp *Bam*HI–*Bgl* II DNA fragment coding for Ag5.2 (15), obtained by PCR, was ligated with plasmid pSMB55 linearized with *Bgl* II. In the new plasmid, designated pSMB70, the Ag5.2 sequence was fused in frame within the *emm6.1* gene. The *emm6.1*–Ag5.2 gene fusion present in pSMB70 included the M protein signal sequence, the sequence coding for the first 122 N-terminal amino acids, and the cell-wall-spanning and anchor sequence (the C-terminal 140 amino acids) of the M6 protein, while the deleted central region of the *emm6.1* gene (538 bp) was replaced by the Ag5.2 sequence (Fig. 1).

Transformation of *S. gordonii*. Recombinant plasmid pSMB70 was used to transform competent *S. gordonii* GP251 cells (Fig. 1). Erythromycin-resistant transformants were tested for both the loss of chloramphenicol resistance and expression of M6 protein. Genetic analysis showed that 100% of erythromycin-resistant, chloramphenicol-sensitive transformants expressed the M6–Ag5.2 fusion protein on the cell surface. One such transformant was chosen as representative and named DM100 (Fig. 1).

Expression of the M6–Ag5.2 Fusion Protein. Expression of the M6–Ag5.2 fusion protein on the surface of *S. gordonii* DM100 was verified by immunofluorescence of whole cells. Recombinant strain DM100 exhibited positive fluorescence when reacted with either Ag5.2-specific monoclonal antibody (Fig. 2) or M6-specific polyclonal antibodies (data not shown), confirming the surface location of the M6–Ag5.2 fusion protein. No fluorescence was observed with wild-type strain GP204. Western blot analysis of the mutanolysin-digested cell wall extract of recombinant DM100 revealed a protein band reactive with both anti-M6 and anti-Ag5.2 antibodies, while extracts from the wild-type *S. gordonii* were negative (data not shown).

Stability of Antigen Expression. The two phenotypes (erythromycin resistance and surface expression of the M6–Ag5.2 fusion protein) were not lost from the strain after *in vitro* passage without antibiotic selection for >50 generations (19). Furthermore, all of the colonies isolated from mice during the 11 weeks of colonization with DM100 expressed both phenotypes.

Mucosal and Systemic Immune Response

Colonization of the Recombinant and Wild-Type Strains in Mice. *S. gordonii* was efficient in colonizing the oral/pharyngeal mucosa of mice after a single oral and intranasal dose. During a 9-week period after inoculation with the recombinant strain, the mean number of positive swabs per mouse was approximately 30% (Fig. 3*A*). There was no significant difference between the colonization of the recombinant and wild-type strains. The highest levels of colonization with recombinant DM100 were observed during the first 3 weeks of colonization (>60% of animals colonized) (Fig. 3*B*). Nine percent of animals were still colonized by 11 weeks (Fig. 3*B*).

Sites of Colonization. Animals colonized with recombinant bacteria were sacrificed at different times and oral and gastrointestinal surfaces analyzed for the presence of the recom-

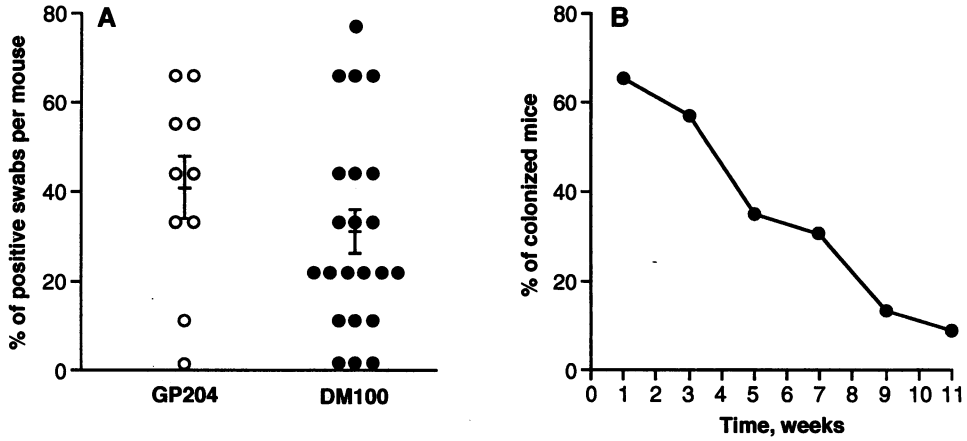

FIG. 3. Oral/pharyngeal colonization of mice with *S. gordonii*. (*A*) Percentage of positive swabs per mouse in a 9-week period following a single oral/intranasal inoculum with wild-type strain GP204 (○) and recombinant strain DM100 (●). Each dot represents an individual mouse (vertical bar = mean ± SEM). (*B*) Percentage of mice colonized with recombinant strain DM100 (●) ($n = 23$). Animals exhibiting one positive swab over a 2-week period were considered positive.

binant *S. gordonii*. By day 20 the organisms primarily colonized the tongue and hard palate in >75% of the animals. Animals followed for up to 40 days showed the same pattern of colonization.

Secretory IgA Response to Ag5.2. Mice colonized with DM100 showed a significant increase of Ag5.2-specific salivary IgA in relation to control mice colonized with wild-type strain GP204 or mice inoculated with killed bacteria (Fig. 4*A*). Antigen-specific IgA was found to be significantly elevated at week 4 (>2% of total IgA) and levels were maintained up to 11 weeks. No significant levels of Ag5.2-specific IgA could be detected in saliva samples from mice inoculated s.c. with DM100 emulsified with Freund's adjuvant.

At week 11, significant levels of Ag5.2-specific IgA were detected in lung lavages of mice colonized with DM100 compared to mice colonized with wild-type GP204 (Fig. 4*B*). No significant levels of Ag5.2-specific IgA were observed in lung lavages from mice inoculated with killed recombinant bacteria or mice colonized with wild-type strain GP204. As with the salivary response, levels of Ag5.2-specific IgA in lung lavages from mice inoculated s.c. with DM100 emulsified with Freund's adjuvant were not significant (Fig. 4*B*).

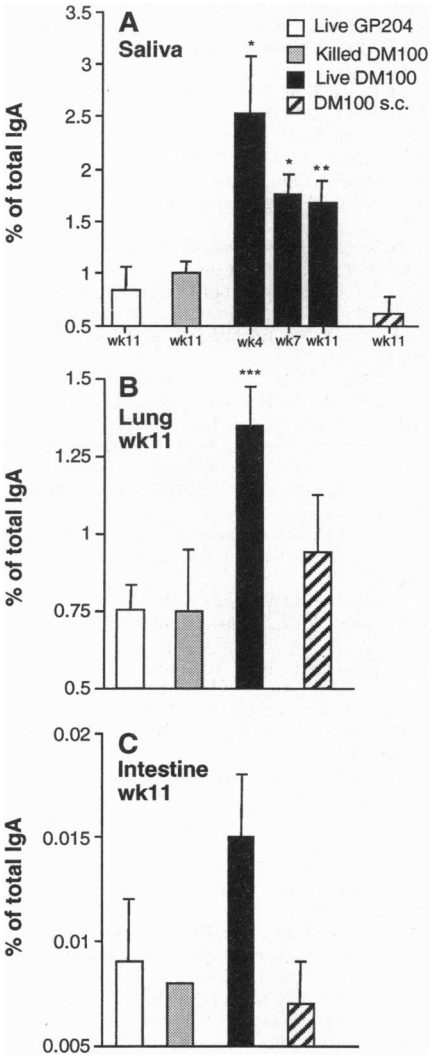

FIG. 4. Secretory IgA response to Ag5.2 in saliva (*A*) and lung (*B*) and intestinal lavages (*C*). Mice were inoculated orally/intranasally with live wild-type GP204, killed recombinant DM100, live recombinant DM100, or inoculated s.c. with killed recombinant DM100. Saliva samples from animals colonized with live DM100 were collected at 4, 7 (n = 6), and 11 weeks (n = 23), and at 11 weeks from animals colonized or injected with the other organisms. Lung and intestinal samples were collected at 11 weeks (n = 23). Samples were tested individually; results are expressed as percent of Ag5.2-specific IgA versus total IgA. Ag5.2-specific IgA in pooled preimmune saliva were equivalent to levels found in GP204-colonized animals (0.46% ± 0.2%). The mean and SEM are indicated. *, **, and *** indicate significant differences between responses of mice inoculated with recombinant DM100 and wild-type GP204 at $P < 0.01$, $P < 0.05$, and $P < 0.001$ levels, respectively.

In contrast to these findings, intestinal lavages exhibited low levels (≈1/100th those of saliva and lung samples) of Ag5.2-specific IgA when compared with the total IgA detected in these samples (Fig. 4*C* and Table 1). Though a small increase was observed in Ag5.2-specific IgA in the intestinal lavages from mice colonized with recombinant bacteria, there was no statistical difference when compared with mice colonized with wild-type GP204.

A positive correlation was observed between levels of Ag5.2-specific IgA detected in both lung lavage and saliva from each mouse ($P < 0.05$), while no correlation was found between these and intestinal samples. More important, we also found a correlation ($P < 0.05$) between the level of antigen-specific IgA detected in lung and saliva and the percent of positive swabs in each mouse.

Serum IgG Response to Ag5.2. Mice colonized with DM100 had a significant Ag5.2-specific serum IgG level compared with control mice colonized with strain GP204 (Fig. 5). Ag5.2-specific IgG was detected between 4 and 7 weeks after inoculation and continued to increase to 11 weeks. Mice inoculated s.c. once at day 0 with strain DM100 emulsified with complete Freund's adjuvant showed a serum IgG titer and time course comparable to that detected in colonized animals (Fig. 5). Mice inoculated orally and intranasally with killed DM100 exhibited no serum IgG response. When animals colonized for 11 weeks were boosted s.c. with purified Ag5.2 in complete adjuvant, a significant increase of Ag5.2-specific IgG was induced (data not shown).

DISCUSSION

Whereas Gram-negative bacteria (10, 23, 24), mycobacteria (25), and viruses (26, 27) have been used as live vectors to deliver foreign antigens to a mammalian host for the purpose of antibody induction, Gram-positive organisms have only recently been exploited for this purpose (12, 28). In the present study, the human oral commensal *S. gordonii* was engineered to express a heterologous surface antigen and used to induce an immune response following oral colonization. Unlike certain other live vaccine vector systems, where the recombinant antigen is expressed from an extrachromosomal element, chromosomal integration in our system assured the stability of the expressed antigen both *in vitro* and *in vivo*. This was verified by the fact that organisms isolated from colonized mice after 11 weeks still expressed the surface allergen.

Compared with other live bacterial systems, where the foreign antigen is either retained in the cytoplasm, translocated to the periplasm, or secreted, the Gram-positive vector described here delivers and anchors the foreign antigen to the cell surface (12). To date, molecules of 15 (16), 98 (12), and 204 amino acids (this study) have been successfully surface expressed on *S. gordonii*. If required, the attachment machinery may be modified, allowing the recombinant molecule to be

Table 1. Range of total IgA concentrations in samples collected from mice

Sample	No. of Samples	Total IgA, μg/ml
Saliva	59	1.9 ± 0.2
Lung lavage	47	2.6 ± 0.2
Intestinal lavage	47	70.4 ± 6.4

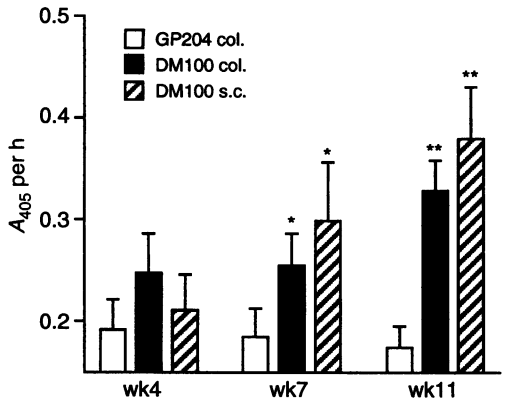

FIG. 5. Serum IgG response. Time course of Ag5.2-specific serum IgG in mice colonized (n = 23) or inoculated s.c. (n = 10) with recombinant DM100 and mice colonized with wild-type GP204 (n = 10). Each value is the mean ± SEM of samples tested individually. * and ** indicate significant differences between responses in mice inoculated with live recombinant *S. gordonii* DM100 and wild-type GP204 at $P < 0.05$ and $P < 0.001$ levels, respectively.

secreted by the commensal (14). Since the attachment motif for surface proteins is highly conserved in Gram-positive bacteria (13), a wide variety of commensals may be engineered for surface expression and used to deliver antigens to respiratory, intestinal, or vaginal sites. Additionally, because the recombinant antigen is complexed to the Gram-positive peptidoglycan (18), a natural adjuvant, the response to the antigen would be enhanced (29).

The presence of secretory IgA at mucosal surfaces is effective in preventing microbial infections (1, 30). Because most human pathogens enter the host through the mucosa, protection from such pathogens may be maximized by employing vaccine strategies that induce an immune response at the site of infection. The mucosal immune response induced after oral/pharyngeal colonization with the recombinant commensal showed significant levels of Ag5.2-specific IgA in pulmonary lavages and saliva. A positive correlation between saliva and lung lavage was noted when the specific IgA was normalized to the total IgA, suggesting a relation between these two secretions (31). Significantly lower levels of Ag5.2-specific IgA could be detected in the intestinal fluid, indicating a higher local induction of the respiratory mucosal immune response after oral colonization. These data support the concept that presentation of the antigen at a specific mucosal site results in higher levels of IgA at that site in comparison with distal mucosal surfaces (7, 8). Mice inoculated orally and intranasally with killed recombinant bacteria showed no systemic or mucosal immune response, suggesting that the recombinant bacteria do not act as passive carriers for the antigen but the prolonged exposure to the antigen following colonization was required to induce the observed immune response. Furthermore, since *S. gordonii* is a human commensal, we expect that in humans the colonization would be prolonged (32), resulting in a sustained immune response.

Although the immune response to commensal bacteria in humans is poorly understood, mammals do in fact develop mucosal and serum antibodies after colonization by certain commensals (33, 34). While these antibodies, for reasons not well understood, do not clear the commensal, it would be expected that such organisms expressing recombinant "foreign" surface molecules would be similarly processed, resulting in the development of an immune response to the recombinant antigen.

The prolonged exposure of the immune system to a recombinant antigen achieved by the stable colonization of a recombinant commensal after a single immunizing dose, can represent a safe and efficient way of overcoming the need for repeated doses of antigen. The induction of a local immune response at the site normally invaded by a pathogen coupled with a boostable systemic response may be a more natural and thus effective method to prevent infections that initiate at mucosal surfaces.

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1. Kraehenbuhl, J. & Neutra, M. R. (1992) *Physiol. Rev.* **72,** 853–879.
2. Mestecky, J. & McGhee, J. R. (1987) *Adv. Immunol.* **40,** 153–245.
3. Childers, N. K., Bruce, M. G. & McGhee, J. R. (1989) *Annu. Rev. Microbiol.* **43,** 503–536.
4. Mazanec, M. B., Nedrud, J. G., Kaetzel, C. S. & Lamm, M. E. (1993) *Immunol. Today* **14,** 430–435.
5. Michetti, P., Mahan, M. J., Slauch, J. M., Mekalanos, J. J. & Neutra, M. R. (1992) *Infect. Immun.* **60,** 1786–1792.
6. Mestecky, J. (1987) *J. Clin. Immunol.* **7,** 265–276.
7. Nedrud, J. G., Liang, X., Hague, N. & Lamm, M. E. (1987) *J. Immunol.* **139,** 3484–3492.
8. Haneberg, B., Kendall, D., Amerongen, H. M., Apter, F. M., Kraehenbuhl, J. & Neutra, M. R. (1994) *Infect. Immun.* **62,** 15–23.
9. Cardenas, L. & Clements, J. D. (1992) *Clin. Microbiol. Rev.* **5,** 328–342.
10. Tacket, C. O., Hone, D. M., Curtiss, R., III, Kelly, S. M., Losonsky, G., Guers, L., Harris, A. M., Edelman, R. & Levine, M. M. (1992) *Infect. Immun.* **60,** 536–541.
11. Tartaglia, J., Perkus, M. E., Taylor, J., Norton, E. K., Audonnet, J., Cox, W. I., Davis, S. W., van der Hoeven, J., Meignier, B., Riviere, M., Languet, B. & Paoletti, E. (1992) *Virology* **188,** 217–232.
12. Pozzi, G., Contorni, M., Oggioni, M. R., Manganelli, R., Tommasino, M., Cavalieri, F. & Fischetti, V. A. (1992) *Infect. Immun.* **60,** 1902–1907.
13. Fischetti, V. A., Pancholi, V. & Schneewind, O. (1990) *Mol. Microbiol.* **4,** 1603–1605.
14. Schneewind, O., Model, P. & Fischetti, V. A. (1992) *Cell* **70,** 267–281.
15. Fang, K. S. Y., Vitale, M., Fehlner, P. & King, T. P. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 895–899.
16. Pozzi, G., Oggioni, M. R., Manganelli, R., Medaglini, D., Fischetti, V. A., Fenoglio, D., Valle, M. T., Kunkl, A. & Manca, F. (1994) *Vaccine* **12,** 1071–1077.
17. Hollingshead, S. K., Fischetti, V. A. & Scott, J. R. (1986) *J. Biol. Chem.* **261,** 1677–1686.
18. Pancholi, V. & Fischetti, V. A. (1988) *J. Bacteriol.* **170,** 2618–2624.
19. Pozzi, G., Oggioni, M. R., Manganelli, R. & Fischetti, V. A. (1992) *Res. Microbiol.* **143,** 449–457.
20. Pozzi, G., Musmanno, R. A., Lievens, P. M., Oggioni, M. R., Plevanl, P. & Manganelli, R. (1990) *Res. Microbiol.* **141,** 659–670.
21. Manjula, B. N., Schmidt, M. L. & Fischetti, V. A. (1985) *Infect. Immun.* **50,** 610–613.
22. Fischetti, V. A. & Windels, M. (1988) *J. Immunol.* **141,** 3592–3599.
23. Doggett, T. A., Jagusztyn-Krynicka, E. K. & Curtiss, R., III (1993) *Infect. Immun.* **61,** 1859–1866.
24. Mekalanos, J. J. (1992) *Adv. Exp. Med. Biol.* **327,** 43–50.
25. Stover, C. K., de la Cruz, V. F., Fuerst, T. R., Burlein, J. E., Benson, L. A., Bennett, L. T., Bansal, G. P., Young, J. F., Lee, M. H., Hatfull, G. F., Snapper, S. B., Barletta, R. G., Jacobs, W. R., Jr., & Bloom, B. R. (1991) *Nature (London)* **351,** 456–460.
26. Moss, B. (1991) *Science* **252,** 1662–1667.
27. Tartaglia, J. & Paoletti, E. (1990) in *Immunochemistry of Viruses II,* eds. van Regenmorterl, M. H. V. & Neurath, A. R. (Elsevier, Amsterdam), pp. 125–151.
28. Hansson, M., Stahl, S., Nguyen, T. N., Bachi, T., Robert, A., Binz, H., Sjolander, A. & Uhlen, M. (1992) *J. Bacteriol.* **174,** 4239–4245.
29. Jolivet, M. E., Audibert, F. M., Gras-Masse, H., Tartar, A. L., Schlesinger, D. H., Wirtz, R. & Chedid, L. A. (1987) *Infect. Immun.* **55,** 1498–1502.
30. Bessen, D. & Fischetti, V. A. (1988) *J. Exp. Med.* **167,** 1945–1950.
31. Steffen, M. J. & Ebersole, J. L. (1992) *Infect. Immun.* **60,** 337–344.
32. Svanberg, M. & Westergren, G. (1986) *Arch. Oral Biol.* **31,** 1–4.
33. Warner, L., Ermak, T. & Griffiss, J. M. (1987) *Adv. Exp. Med. Biol.* **216,** 959–964.
34. Gold, R., Goldschneider, I., Lepow, M. L., Draper, T. F. & Randolph, M. (1978) *J. Infect. Dis.* **137,** 112–121.
35. King, T. P., Kochoumian, L. & Lu, G. (1995) *J. Immunol.* **154,** 577–584.